Placental promoter methylation of DNA repair genes and prenatal exposure to particulate air pollution: an ENVIRONAGE cohort study

Kristof Y Neven, Nelly D Saenen, Letitzia Tarantini, Bram G Janssen, Wouter Lefebvre, Charlotte Vanpoucke, Valentina Bollati, Tim S Nawrot

Summary

Background Exposure to particulate air pollution has been linked with risk of carcinogenesis. Damage to repair pathways might have long-term adverse health effects. We aimed to investigate the association of prenatal exposure to air pollution with placental mutation rate and the DNA methylation of key placental DNA repair genes.

Methods This cohort study used data from the ongoing ENVironmental Influence *ON* early AGEing (ENVIRONAGE) birth cohort, which enrols pairs of mothers and neonates (singleton births only) at the East-Limburg Hospital (Genk, Belgium). Placental DNA samples were collected after birth. We used bisulfite-PCR-pyrosequencing to investigate the mutation rate of *Alu* (a marker for overall DNA mutation) and DNA methylation in the promoter genes of key DNA repair and tumour suppressor genes (*APEX1*, *OGG1*, *PARP1*, *ERCC1*, *ERCC4*, *p53*, and *DAPK1*). We used a high-resolution air pollution model to estimate exposure to particulate matter with a diameter less than 2·5 µm ($PM_{2·5}$), black carbon, and NO_2 over the entire pregnancy on the basis of maternal address. *Alu* mutation was analysed with a linear regression model, and methylation values of the selected genes were analysed in mixed-effects models. Effect estimates are presented as the relative percentage change in methylation for an ambient air pollution increment of one IQR (ie, the difference between the first and third quartiles of exposure in the entire cohort).

Findings 500 biobanked placental DNA samples were randomly selected from 814 pairs of mothers and neonates who were recruited to the cohort between Feb 1, 2010, and Dec 31, 2014, of which 463 samples met the pyrosequencing quality control criteria. IQR exposure increments were 3·84 µg/m³ for $PM_{2·5}$, 0·36 µg/m³ for black carbon, and 5·34 µg/m³ for NO_2. Among these samples, increased *Alu* mutation rate was associated with greater exposure to $PM_{2·5}$ (r=0·26, p<0·0001) and black carbon (r=0·33, p<0·0001), but not NO_2. Promoter methylation was positively associated with $PM_{2·5}$ in *APEX1* (7·34%, 95% CI 0·52 to 14·16, p=0·009), *OGG1* (13·06, 3·88 to 22·24, p=0·005), *ERCC4* (16·31%, 5·43 to 27·18, p=0·01), and *p53* (10·60%, 4·46 to 16·74, p=0·01), whereas promoter methylation of *DAPK1* (–12·92%, –22·35 to –3·49, p=0·007) was inversely associated with $PM_{2·5}$ exposure. Black carbon exposure was associated with elevated promoter methylation in *APEX1* (9·16%, 4·06 to 14·25, p=0·01) and *ERCC4* (27·56%, 17·58 to 37·55, p<0·0001). Promoter methylation was not associated with pollutant exposure in *PARP1* and *ERCC1*, and NO_2 exposure was not associated with methylation in any of the genes studied.

Interpretation Transplacental in-utero exposure to particulate matter is associated with an increased overall placental mutation rate (as measured with *Alu*), which occurred in concert with epigenetic alterations in key DNA repair and tumour suppressor genes. Our results suggest that exposure to air pollution can induce changes to fetal and neonatal DNA repair capacity. Future studies will be essential to elucidate whether these changes persist and have a role in carcinogenic insults later in life.

Funding European Research Council and the Flemish Scientific Fund.

Lancet Planet Health 2018; 2: e174–83

See **Comment** page e149

Centre for Environmental Sciences, Hasselt University, Hasselt, Belgium (K Y Neven MSc, N D Saenen PhD, B G Janssen PhD, Prof T S Nawrot PhD); **EPIGET—Epidemiology, Epigenetics and Toxicology Lab, Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy** (L Tarantini MSc, Prof V Bollati PhD); **Flemish Institute for Technological Research, Mol, Belgium** (W Lefebvre PhD); **Belgian Interregional Environment Agency, Brussels, Belgium** (C Vanpoucke MSc); **and Environment & Health unit, Leuven University, Leuven, Belgium** (Prof T S Nawrot)

Correspondence to: Prof Tim Nawrot, Centre for Environmental Sciences, Hasselt University, Hasselt B-3500, Belgium
tim.nawrot@uhasselt.be

Introduction

The International Agency for Research on Cancer (IARC) has classified ambient air pollution as a group 1 carcinogen in human beings.[1] Particulate matter, defined as ambient particles with a diameter smaller than 2·5 µm ($PM_{2·5}$), is a key component of air pollution. Black carbon is a form of combustion-related $PM_{2·5}$ air pollution.[2] During in-utero development, the fetus has heightened susceptibility to the effects of carcinogenic air pollutant exposure. According to evidence from an ex-vivo human placental perfusion model, particles with a diameter less than 240 nm are able to travel across the placenta to the fetal side.[3]

Altered expression of tumour suppressor genes or oncogenes during in-utero development, if persistent, could result in carcinogenic insults. The tumour suppressor p53 becomes activated after DNA damage, oncogene activation, or hypoxia and can induce cellular responses such as apoptosis, cell-cycle arrest, senescence, and autophagy.[4] Death-associated protein

Research in context

Evidence before this study

Between Aug 21, 2015, and March 31, 2017, we searched PubMed and Web of Science for longitudinal studies that investigated the effects of prenatal exposure to particulate air pollution on the epigenetic methylation levels of the promoter regions of DNA repair genes and tumour suppressor genes. We used the search terms ("particulate matter" or "air pollution") plus "methylation" plus ("DNA repair" or "tumour suppressor") plus ("gestational" or "prenatal" or "pregnancy"). Although DNA repair genes are crucial in maintaining a healthy cellular environment, and while particulate air pollution is omnipresent, no cohort studies of mothers and neonates so far have investigated the association between prenatal exposure to air pollution and placental methylation of DNA repair and tumour suppressor genes as a potential foundation of transplacental carcinogenesis.

Added value of this study

Our results suggest that prenatal exposure to particulate matter, even at a level below that recommended by European Union guidelines (ie, average exposure during pregnancy of 13·5 μg/m³ compared with the annual average threshold of 25·0 μg/m³) and black carbon is associated with altered promoter methylation of key DNA repair and tumour suppressor genes, as well as an increased placental mutation rate. Our study population is representative for the reproductive segment of the general population (Flanders, Belgium) and we used a standardised fine-scale exposure assessment with exposure levels similar to those in other European cohorts.

Implications of all the available evidence

During in-utero development, the fetus is susceptible to transplacental exposure to carcinogenic air particles. Even below European Union air pollution thresholds, exposure to particulate matter seemed to be associated with an increased placental mutation rate. This increased mutation rate occurred in concert with epigenetic alterations in key DNA repair and tumour suppressor genes in the placenta. Our findings therefore suggest that prenatal air pollution is able to affect mechanisms that could cause carcinogenic insults, which, if persistent, might have potential clinical implications later in life. It is therefore essential to investigate the persistence of these epigenetic changes over the entire life course.

kinase 1 (DAPK1) is involved in similar biological functions, including programmed cell death and autophagy.[5] The correct functioning of these tumour suppressor genes is essential to maintain the healthy cellular environment through the elimination of cells with a high mutational burden.

DNA damage can be repaired with specialised repair pathways, including the base excision repair (BER) and nucleotide excision repair (NER) pathways (figure 1). The BER pathway repairs damage caused by excessive production of the spontaneous decay products that are formed within DNA and damage induced by natural endogenous chemicals such as reactive oxygen species.[6] The BER pathway can function via a short-patch repair or a long-patch repair, depending on various factors such as the specificity of the initiating glycosylase and the availability of oxoguanine glycosylase 1 (OGG1).[7] Other essential proteins in BER include AP endonuclease 1 (APEX1) and poly(ADP-ribose) polymerase 1 (PARP1). Whereas the BER pathway focuses mostly on mending damage from endogenously generated products, the NER pathway is specialised for repairing damage from exogenous sources, such as ultraviolet light exposure and carcinogenic compounds.[6,8] The complex of excision repair complementing factors 1 and 4 (*ERCC1-4*) is an important incision complex in the NER pathway and is required to incise the DNA strand at the correct location. Because of their importance in DNA repair, deficiencies in the BER and NER pathways have been directly linked to the development of cancer in both mice and human beings.[8,9]

See Online for appendix

However, despite the importance of DNA repair genes and the omnipresence of ambient air pollution, to our knowledge, no epidemiological studies so far have investigated the association between prenatal exposure to $PM_{2.5}$, black carbon, or NO_2 and the methylation of DNA repair and tumour suppressor genes in the placenta and the placental mutation rate. We postulated that prenatal exposure to air pollution during the entire pregnancy might be associated with placental DNA mutations and alterations in the methylation of the promoter regions of genes involved in the DNA repair and tumour suppressor pathways.

Materials and methods

Study design and participants

This cohort study used data from the ongoing ENVironmental Influence *ON* early AGEing (ENVIR*ON*AGE) birth cohort.[10] The study protocol was approved by the Ethics Committee of Hasselt University and East-Limburg Hospital in Genk (Belgium) and was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participating mothers.

Pairs of mothers and neonates (singleton births only) were recruited into the cohort at the East-Limburg Hospital (Genk, Belgium) during weekends. The catchment area of the hospital where we recruited our participants includes the province of Limburg (Flanders, Belgium) and combines urban, suburban, and rural areas, with population densities of the municipalities ranging from 82 to 743 inhabitants per km² (appendix).

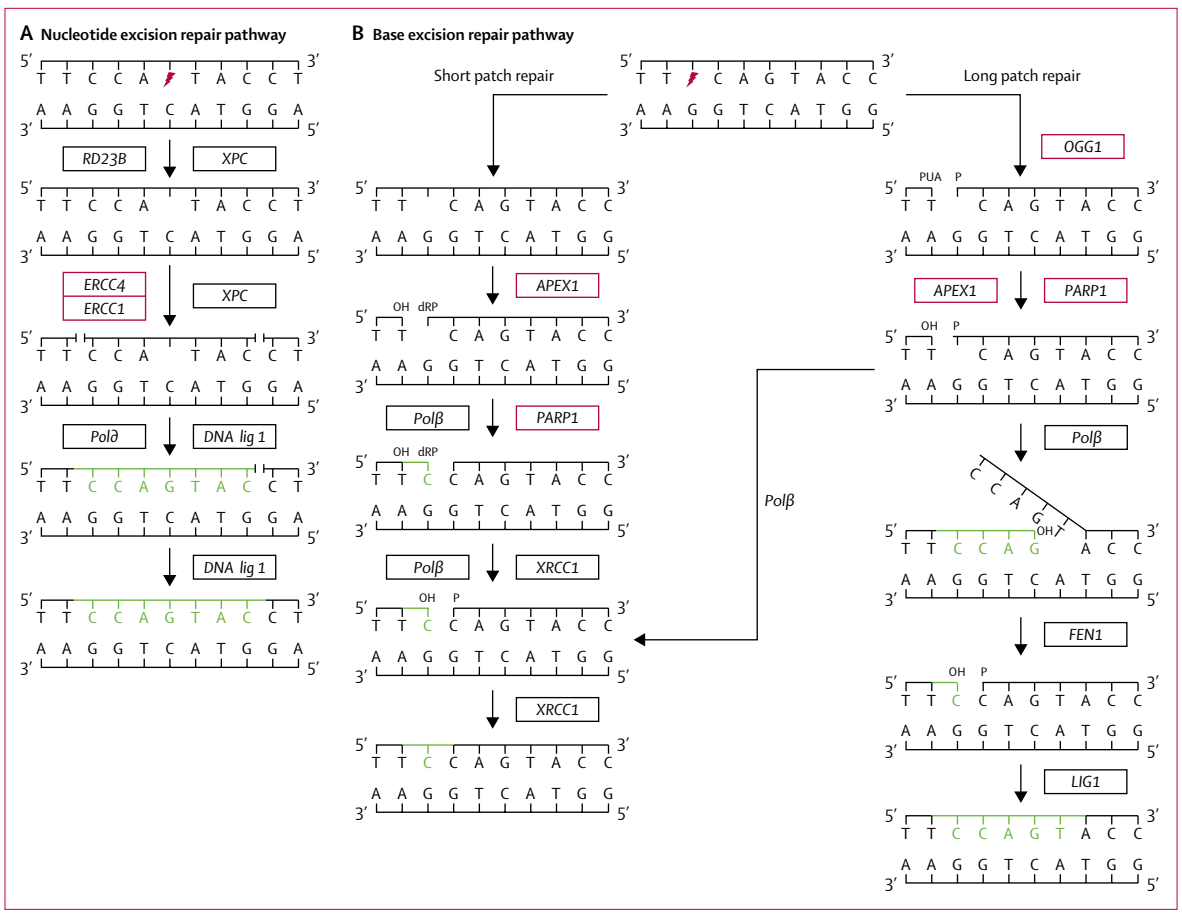

Figure 1: **DNA repair pathways**
The nucleotide excision repair pathway (A) and the base excision repair pathway (B) are shown, with the most important genes in the pathways indicated. The genes investigated in the present study are highlighted with red boxes. dRP=5′ deoxyribose phosphate. PUA= β-unsaturated aldehyde

The inclusion criteria were that mothers were able to complete questionnaires in Dutch, provide written consent, and did not have planned caesareans. Participants were recruited equally during all seasons of the year. Midwives recorded the main reasons for non-participation (failure to ask about participation, communication barriers, or complications during labour). The questionnaires provided detailed medical and lifestyle data, including maternal age, maternal education, maternal smoking status, ethnicity, residential address, maternal pre-pregnancy body-mass index (BMI), and parity. Full details of the data collected have been published previously.[10] Maternal education was coded as low (no diploma or primary school), middle (high school), and high (college or university degree). Smoking status was classified as non-smokers, stopped smoking before pregnancy, and current smokers (smoked during pregnancy). Ethnicity was classified on the basis of the native country of the neonate's grandparents as either European (at least two grandparents were European) or non-European (at least three grandparents were of non-European origin), and parity was coded as primiparous (first child), secundiparous (second child), and multiparous (third or later child).

Sample collection and testing

Placenta were deep frozen within 10 min after delivery. After thawing, placental tissue biopsies were taken at a standardised location, as detailed by Janssen and colleagues.[10] In short, on the fetal side of the placenta, the largest umbilical cord artery was used as a waypoint to identify the entry point. Biopsies were taken at a distance of 4 cm from the umbilical cord and 1–1·5 cm below the chorioamniotic membrane, and were sampled towards the umbilical cord. Biopsies were thoroughly washed with phosphate-buffered saline, snap frozen with liquid nitrogen, and stored at –80°C until DNA extraction.

Genomic DNA was isolated from placental tissue with the QIAamp DNA mini kit (Qiagen, Venlo, Netherlands) in accordance with the manufacturer's instructions. An aliquot of 0·5 µg DNA was then treated using the EZ-96 DNA Methylation-Gold Kit (Zymo Research, CA, USA) in accordance with the manufacturer's protocol. The final elution was done in 30 µL M-elution buffer

For the **UCSC Genome Browser** see http://genome.ucsc.edu/

(Zymo Research). Bisulfite-treated DNA was stored at –80°C until PCR amplification.

We used the UCSC Genome Browser and research literature to identify transcriptionally important CpG sites within the promoter regions of *APEX1, PARP1, OGG1, ERCC1, ERCC4, p53*, and *DAPK1* (appendix).[11,12] Data were derived from the hg38 (GRCh38/hg38) database for all interrogated genes, except for *PARP1*, for which the hg19 (GRCh37/hg19) database was consulted, in accordance with the work of Alvarado-Cruz and colleagues.[12] In 2004, Yang and colleagues investigated a pool of *Alu* repetitive elements, amplified by means of primers, the targets of which are located in a highly conserved region of the *Alu* retrotransposon.[13] This method was shown to be able to quantify, in parallel, both *Alu* methylation (as an estimate of global methylation) and *Alu* mutation as an estimate of global mutation.[14] We applied this method to obtain a mutation marker, accounting for simple C to T or G to A point mutations.

The PCR products of the regions of interest were amplified from bisulfite-modified DNA by use of GoTaq HotStart Green Master Mix (Promega, Madison, WI, USA). The PCR product was stored at 4°C immediately after its generation until pyrosequencing measurements were done, which occurred a maximum of 48 h after the PCR product was generated. Detailed information about gene assembly and primer sequences is shown in the appendix.

The final PCR product was purified with Streptavidin Sepharose HP beads (Amersham Biosciences, Uppsala, Sweden), to which the biotin-labelled PCR product was bound. The beads containing the PCR product were purified in 70% ethanol, washed, denatured in a 0·2 M NaOH solution, and washed in washing buffer with the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Westborough, MA, USA), in accordance with the manufacturer's instructions. Afterwards, 0·3 µM gene-specific pyrosequencing primer was annealed to the single-stranded PCR product and pyrosequencing was done with a Pyromark Q9 MD Pyrosequencing System (Qiagen, Germantown, MD, USA). The degree of methylation was presented as the percentage of methylated cytosines divided by the sum of both methylated and unmethylated cytosines (% 5-mC). We duplicated the pyrosequencing runs from the same subset of samples to determine the reproducibility of the PCR and pyrosequencing assays

Prenatal exposure assessment

The maternal residential address was used to interpolate regional $PM_{2·5}$, black carbon, and NO_2 concentrations in µg/m³, with interpolation based on the Kriging spatial temporal interpolation method described by Janssen and colleagues.[15] This method uses satellite images from the CORINE land cover dataset, which was initiated in the European Union and provides land cover data to interpolate the pollution data provided by the official Belgian fixed monitoring network, in combination with a dispersion model.[16,17] This model chain provides interpolated air pollution values from the Belgian telemetric air quality networks, point sources (ie, industry), and line sources (ie, highways), on a dense, irregular receptor point grid (maximum grid cell size of 25 m by 25 m). The overall model performance was assessed by leave-one-out cross-validation, including 14 monitoring stations for black carbon, 44 for NO_2, and 34 for $PM_{2·5}$. The validation statistics of the interpolation tool explained more than 74% of the temporal and spatial variability in Flanders for black carbon, 78% for NO_2, and 80% for $PM_{2·5}$.[16,18] By use of the described model, daily air pollution levels were modelled for each mother and covered the total pregnancy period. The daily values were averaged for specific time windows during the pregnancy: first trimester (ie, date of conception until 13 weeks of pregnancy), second trimester (ie, 14 weeks until 26 weeks of pregnancy), third trimester (ie, 27 weeks of pregnancy until delivery), and the entire period of pregnancy, from the date of conception until the date of delivery. For mothers who moved during pregnancy (n=56), we calculated the specific exposures, allowing for the changes in address during pregnancy (based on the daily exposure levels at the different residential addresses). The date of conception was estimated from the first day of the mother's last menstrual period, combined with the first ultrasound exam.

Statistical analysis

We used SAS software version 9.4 to do the statistical analysis. Categorical data were presented as frequency and percentage, whereas continuous data were presented as mean (SD). In a subset of 14 placentas, from which samples from all four quadrants were taken, the intra-placental variability was assessed with the intraclass correlation coefficient. *Alu* mutation was analysed in a linear regression model, and the methylation values of the selected genes were analysed in mixed-effects models. The normality of the data was tested with both the Shapiro-Wilk statistic and quantile-quantile plots. All models were adjusted for the neonate's sex, ethnicity, and parity and maternal age, education, smoking habits, and pre-pregnancy BMI, as well as gestational age, season at delivery, and batch effect. To obtain a relevant exposure change per exposure, the effect estimates of the mutation marker and genes are presented as a relative percentage change in methylation level for an ambient air pollution ($PM_{2·5}$, black carbon, and NO_2) exposure increment of one IQR (ie, the difference between the first and third quartiles of exposure in the entire cohort) for different exposure windows (total pregnancy and trimester specific exposure).

In sensitivity analyses, we investigated the associations between the placental DNA repair gene methylation and $PM_{2·5}$ or black carbon exposure during the entire pregnancy while excluding preterm births, mothers with

gestational diabetes, gestational hypertension, or maternal smoking status. Additionally, we added the apparent mean temperature and relative humidity during the third trimester as adjustments in the sensitivity model.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

In the present study, 500 biobanked placental DNA samples were randomly selected from 814 pairs of mothers and neonates who were recruited to the cohort between Feb 1, 2010, and Dec 31, 2014. After the exclusion of samples that did not meet the pyrosequencing quality control criteria (ie, those that failed the reference sequence [n=12], and those with low signal-to-noise ratios [n=25], we included 463 pairs in our analyses. The datasets analysed during the current study are available from the corresponding author on reasonable request.

Sociodemographic and clinical characteristics of the 463 mother and neonate pairs are presented in table 1. Mean maternal age was 29·2 years (SD 4·6). Mean pre-pregnancy BMI was 24·2 kg/m² (4·5) and most (246 [53%]) mothers had received a higher education. 60 (13%) of the mothers reported smoking during the pregnancy, whereas 288 (62%) had never smoked. 76 mothers (16%) consumed alcohol during the pregnancy. Of the neonates, 234 (51%) were boys and the mean gestational age was 39·2 weeks (SD 1·3). Mean birthweight was 3403 g (434) and mean length was 50·3 cm (2·0). Most neonates (254 [55%]) were primiparous or secundiparous (163 [35%]). 415 neonates (90%) had at least two European grandparents. The participation rate was spread evenly over the recruitment period (data not shown).

During the entire pregnancy, mean exposure was 13·61 μg/m³ (IQR 11·4–15·3) for ambient $PM_{2.5}$, 0·90 μg/m³ (0·7–1·1) for black carbon, and 18·57 μg/m³ (15·9–21·3) for NO_2. Table 2 shows the trimester-specific exposures. The observed exposure concentrations overlap with most of the 22 European cohorts within the multicentre ESCAPE project.[19]

Prenatal exposure to $PM_{2.5}$ during the entire pregnancy was positively associated with the mutation rate of *Alu* in the placenta (r=0·26, p<0·0001; figure 2A). After adjustment, we detected a relative increase of 2·91% (95% CI 0·20–5·70, p=0·035) in *Alu* mutation rate for an IQR increment of 3·84 μg/m³ $PM_{2.5}$ during the entire pregnancy. For black carbon, we detected an increase (r=0·33, p<0·0001) in *Alu* mutation rate of 1·97% (95% CI 1·40–2·54, p=0·0007; figure 2B) for an IQR increment of 0·36 μg/m³ during the entire pregnancy. We did not detect a significant association between NO_2 exposure

	Mother and neonate pairs (n=463)
Maternal variables	
Age (years)	29·2 (4·6)
Pre-pregnancy BMI (kg/m²)	24·2 (4·5)
Self-reported tobacco use	
Never smoked	288 (62%)
Smoked before pregnancy	115 (25%)
Smoked during pregnancy	60 (13%)
Alcohol consumption during pregnancy	
None	387 (84%)
≤1 glass per day	68 (15%)
>1 glass per day	8 (2%)
Maternal education*	
Low	47 (10%)
Middle	170 (37%)
High	246 (53%)
Neonate variables	
Gestational age (weeks)	39·2 (1·3)
Birthweight (g)	3403 (434)
Birth length (cm)	50·3 (2·0)
Sex	
Male	234 (51%)
Female	229 (49%)
Ethnicity†	
European	415 (90%)
Non-European	48 (10%)
Parity	
Primiparous	254 (55%)
Secundiparous	163 (35%)
Multiparous	46 (10%)
Season at birth	
Winter (Dec 21 to March 20)	125 (27%)
Spring (March 21 to June 20)	108 (23%)
Summer (June 21 to Sept 22)	108 (23%)
Autumn (Sept 23 to Dec 20)	122 (26%)
DNA methylation (% 5-mC)	
APEX1	1·3 (0·9)
OGG1	0·8 (0·9)
PARP1	1·9 (2·4)
ERCC1	1·7 (2·3)
ERCC4	1·8 (2·3)
p53	4·9 (3·6)
DAPK1	1·1 (1·1)
DNA mutation rate (%)	
Alu	22·9 (5·9)

Data are mean (SD) or n (%). *Maternal education was coded as low (no diploma or primary school), middle (high school), and high (college or university degree). †Classification of ethnicity is based on the native country of the neonates' grandparents as either European (at least two grandparents were European) or non-European (at least three grandparents were of non-European origin).

Table 1: **Clinical and sociodemographic characteristics of the mother and neonate pairs**

and *Alu* mutation rate (r=0·10, p=0·098). The rate of placental mutation in *Alu* was positively associated with placental methylation of *APEX1* (*r*=0·18, p=0·028), *OGG1* (*r*=0·28, p=0·0053), *PARP1* (*r*=0·19, p<0·0001), *ERCC1* (*r*=0·18, p=0·0003), and *ERCC4* (*r*=0·46, p<0·0001). No significant associations were observed between *Alu* mutation and *p53* (*r*=–0·04, p=0·42) or *DAPK1* (*r*=0·03, p=0·55).

We fitted adjusted mixed-effects models to investigate the relationship between ambient air pollution exposure across the entire pregnancy and relative placental methylation levels in the promoter regions of *APEX1*, *OGG1*, *PARP1*, *ERCC1*, *ERCC4*, *p53*, and *DAPK1* (figure 3; appendix). During the entire pregnancy, NO_2 exposure was not significantly associated with any of the investigated DNA repair or tumour suppressor genes (appendix).

In the BER pathway, an IQR increment of 3·84 μg/m³ of $PM_{2·5}$ during the entire pregnancy was associated with an increase in methylation of 7·34% (95% CI 0·52–14·16, p=0·0089) for *APEX1* and 13·06% (3·88 to 22·24, p=0·0054) for *OGG1*. During the same exposure window, an IQR increment of 0·36 μg/m³ of black carbon was associated with an increase in methylation of 9·16% (95% CI 4·06–14·25, p=0·005) for *APEX1*, but was not associated with *OGG1*. *PARP1* promoter methylation was not associated with any types of air pollution exposure.

Within the NER pathway, placental *ERCC4* methylation was increased by 16·31% (95% CI 5·43–27·18, p=0·0034) for an IQR increment of $PM_{2·5}$ and 27·56% (17·58–37·55, p<0·0001) for an IQR increment of black carbon during the entire pregnancy. Placental *ERCC1* methylation was not significantly associated with either $PM_{2·5}$ or black carbon exposure.

For the tumour suppressor genes, an IQR increment of $PM_{2·5}$ exposure was associated with an increase of

	Median (IQR)	IQR increment (third quartile minus first quartile)
$PM_{2·5}$ exposure (μg/m³)		
Trimester 1 (1–13 weeks)	12·12 (9·30–17·44)	8·15
Trimester 2 (14–26 weeks)	11·83 (9·21–16·57)	7·36
Trimester 3 (27 weeks–delivery)	11·73 (8·90–17·09)	8·19
Entire pregnancy	13·61 (11·43–15·27)	3·84
Black carbon exposure (μg/m³)		
Trimester 1 (1–13 weeks)	0·90 (0·63–1·17)	0·54
Trimester 2 (14–26 weeks)	0·90 (0·63–1·13)	0·51
Trimester 3 (27 weeks–delivery)	0·94 (0·65–1·17)	0·52
Entire pregnancy	0·90 (0·74–1·10)	0·36
NO_2 exposure (μg/m³)		
Trimester 1 (1–13 weeks)	18·75 (14·55–22·85)	8·30
Trimester 2 (14–26 weeks)	18·21 (14·26–22·65)	8·39
Trimester 3 (27 weeks–delivery)	18·30 (14·08–23·14)	9·06
Entire pregnancy	18·57 (15·93–21·27)	5·34

$PM_{2·5}$=particulate matter with a diameter <2·5 μm.

Table 2: **Exposure to $PM_{2·5}$, black carbon, and NO_2 by time window**

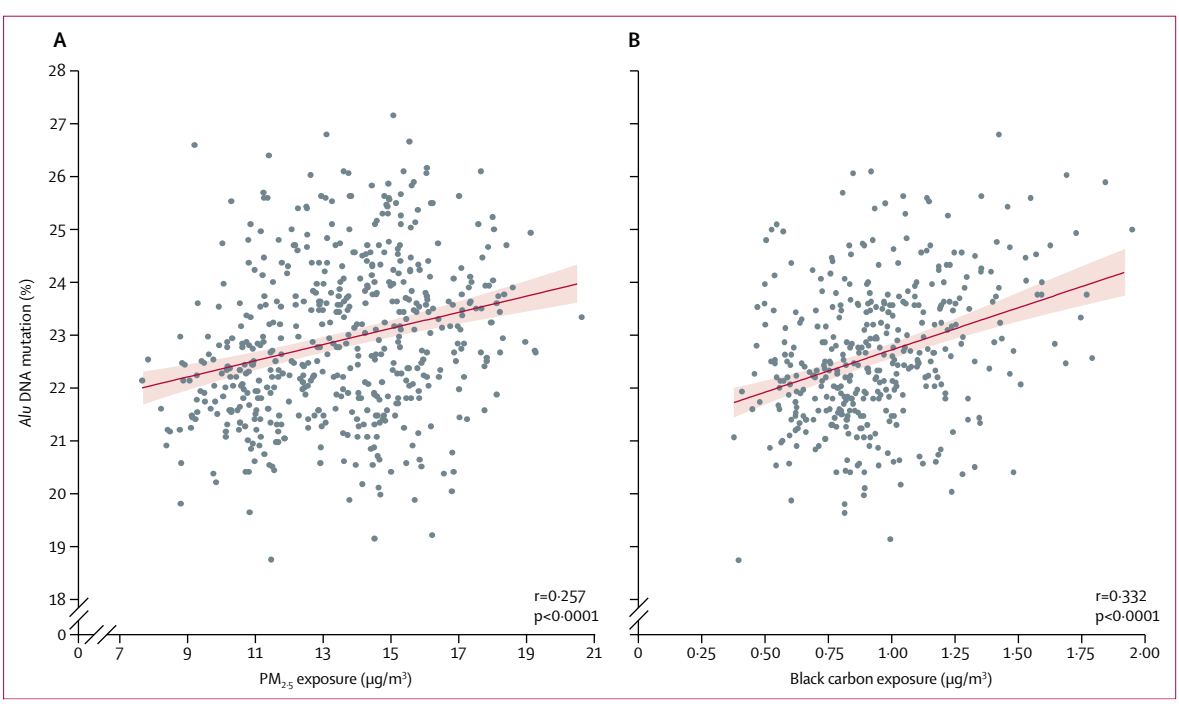

Figure 2: **Correlation between placental *Alu* mutation rate and air pollution exposure**
Plots show the correlation between *Alu* mutation rates as absolute percentages and exposure to (A) $PM_{2·5}$ and (B) black carbon (in μg/m³) during the entire pregnancy. $PM_{2·5}$=particulate matter with a diameter <2·5 μm.

10·60% (95% CI 4·46 to 16·74, p=0·0008) in *p53* methylation, but a decrease of 12·92% (–22·35 to –3·49, p=0·0073) in *DAPK1* methylation. Neither *p53* nor *DAPK1* was significantly associated with black carbon exposure.

In our adjusted trimester-specific analyses, $PM_{2·5}$ exposure during the third trimester was significantly associated with promoter methylation of *APEX1* and *OGG1* (appendix). Furthermore, exposure to $PM_{2·5}$ was significantly associated with *ERCC4* promoter methylation in all trimesters. For the tumour suppressor genes, $PM_{2·5}$ exposure was positively associated with *p53* promoter methylation in the first trimester, whereas exposure in the second trimester was associated with decreased *DAPK1* promoter methylation. We detected no significant associations between $PM_{2·5}$ exposure and *Alu* mutation rate in any trimesters. Exposure to black carbon in the first and second trimesters of pregnancy was positively associated with *ERCC4* promoter methylation, and black carbon exposure in the third trimester was associated with *APEX1* promoter methylation. Exposure to black carbon was positively associated with *Alu* mutation in the second and third trimesters (appendix).

In a sensitivity analysis, we excluded preterm births (n=23), mothers with gestational diabetes (n=28) or pre-eclampsia (a pregnancy disorder characterised by the onset of high blood pressure and proteinuria; n=2), and mothers who smoked during the pregnancy (n=60). These exclusions had no significant effects on the estimated associations between DNA repair gene promoter methylation and $PM_{2·5}$ or black carbon exposure during the entire pregnancy (appendix). We also estimated both ambient temperature and relative humidity in the third trimester of pregnancy for the total population and included these factors in our model (n=463); the inclusion of these factors had no effects on the significance of our results.

In the substudy of 14 placentas, intraplacental variability was 6·32% for *APEX1*, 8·36% for *OGG1*, 11·15% for *PARP1*, 8·04% for *ERCC1*, 6·14% for *ERCC4*, 7·62% for *p53*, 10·42% for *DAPK1*, and 1·37% for *Alu*. Additionally, Pearson's correlation coefficient was used to determine the assay reproducibility in a subset of 14 participants. Assay reproducibility (*r*) was 0·90 for *APEX1*, 0·87 for *OGG1*, 0·87 for *ERCC1*, 0·89 for *ERCC4*, 0·91 for *p53*, and 0·82 for *DAPK1*.

Discussion

In 463 neonates, methylation patterns in the promoter regions of key DNA repair and suppressor genes and the *Alu* mutation rate—a measure of the overall DNA mutation rate—in placental tissue were associated with prenatal exposure to $PM_{2·5}$ and black carbon across the entire pregnancy. Our key findings were that elevated prenatal exposure to $PM_{2·5}$ and black carbon, but not NO_2, was associated with an increased placental

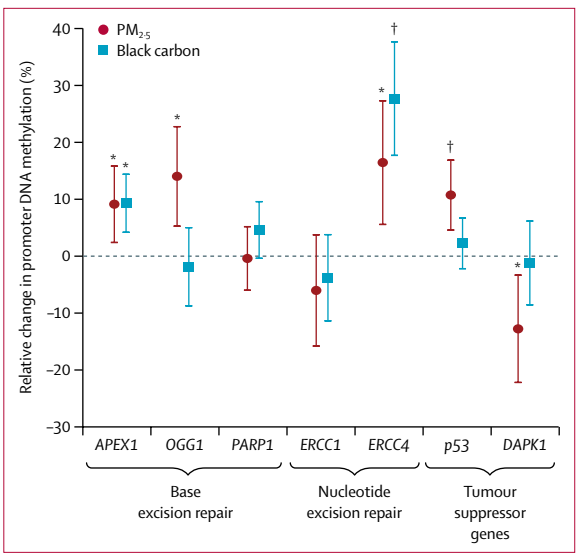

Figure 3: **Pollutant exposure and promoter methylation in DNA repair and tumour suppressor genes**
Effect estimates are presented as the relative change in DNA methylation for an increase of one IQR increment of $PM_{2·5}$ (3·84 μg/m³) or black carbon (0·36 μg/m³) during the entire pregnancy. Estimates are adjusted for neonates' sex and ethnicity, maternal age, education, smoking habits, and pre-pregnancy body-mass index, and gestational age, batch effect, and season at delivery. *p<0·01. †p<0·0001.

Alu mutation rate and increased promoter methylation in key DNA repair genes in the placenta. Additionally, with respect to tumour suppressor genes, elevated exposure to $PM_{2·5}$ was associated with increased promoter methylation of *p53*, but decreased promoter methylation of *DAPK1*. Transplacental exposure to carcinogenic air pollutants from combustion engines is a growing health concern because of the susceptibility of fetuses to the effects of these carcinogens. The specific mechanisms behind fetal developmental programming need to be investigated to unravel the complex interplay between external and biological factors. Doing so could improve understanding of the developmental origins of health and diseases and the relationship between prenatal exposure and cancer risk in later life. Although the observed exposure characteristics were well below those recommended in the European Air Quality Standards (maximum annual $PM_{2·5}$ concentration of 25 μg/m³), these effects were still associated with the methylation level of the investigated genes and the mutation rate.[20]

DNA damage can occur in the form of simple transversion mutations at CpG sites; such mutations make up a quarter of all somatic point mutations in the *p53* gene.[21] Yang and colleagues suggest that CpG sites can be mutated in a unidirectional manner to a TpG or a CpA site.[14] These point mutations could prove to be crucial, as cells grow exponentially during the pregnancy and might therefore be susceptible to an elevated mutation rate compared with that of adult

physiological tissues. Although CpG sites comprise 1% of the genome, they account for roughly 40–70% of all breakpoints in leukaemia-associated genes.[22] We found that the placental mutation rate, measured in repetitive *Alu* sequences, was positively associated with $PM_{2·5}$ and black carbon air pollution. These results suggest that the degree of DNA mutation is heightened within the entire genome because of exposure to ambient air pollution and combustion-related pollutants. This observation is in line with the findings of Perera and colleagues,[23] who showed that transplacental exposure to air pollutants can induce aromatic-DNA adducts in cord blood. Although these mutations were only observed in CpG sites, evidence suggests that the frequency of transversions involving the C at CpG sites is similar to the frequency of transitions at non-CpG sites.[24] According to these data, we could infer that CpG sites in *Alu* sequences could be a representative marker for DNA mutation in the entire genome.

Two key NER genes showed heightened methylation related to elevated prenatal air pollution: *APEX1* and *OGG1*. The promoter regions of genes involved in the BER and NER pathways have been shown to be hypermethylated within cancer cells,[25] which is in line with our finding of increased methylation of these genes in the placenta for neonates with higher prenatal $PM_{2·5}$ and black carbon exposure. *APEX1* can initiate the apurinic/apyrimidinic endodeoxyribonuclease in the BER pathway and has been described as an essential element for survival.[26] Furthermore, *APEX1* has additional roles in controlling the cellular proliferation rate and initiating the repair of oxidatively damaged guanine bases in DNA.[26] This initiation function is shared by *OGG1,* as shown in mice studies[27,28] in which ogg1 was responsible for most BER activity. Shortened lifespan and a tendency towards increased mutation rates in tumours could be attributed to *ogg1* deficiencies.[27] Additionally, mice who were deficient for *ogg1* had impaired neurodevelopment.[28] These findings suggest that the *OGG1* gene is involved in multiple developmental processes. The BER pathway has a strictly organised mode of action to provide an accurate repair mechanism while avoiding the creation of toxic intermediate products.[26] Decreased activity of either *APEX1* or *OGG1* could cause the accumulation of DNA damage or sensitivity to DNA damaging agents. Although we detected increased methylation in both the *APEX1* (for both $PM_{2·5}$ and black carbon) and *OGG1* promoter regions, we found no significant changes in *PARP1* promoter methylation.

Whereas insufficiencies in the BER pathway are mostly associated with risk of lung and colorectal cancer, deficiencies in the NER pathway mainly result in extreme photosensitivity and a predisposition to skin cancer.[29] ERCC1 has been described as the key enzyme in the nucleotide excision repair pathway because it is responsible for catalysing the 5′ cleavage, while ERCC4 provides an additional stabilising function.[30] Importantly, only after the 5′ side is incised can the damaged nucleotide be excised.[31] Our findings suggest that exposure to pollutants could cause the ERCC1-4 complex to lose the stabilising function of the ERCC4 protein because of a decreased expression, as shown by the increased promoter methylation of *ERCC4*. *ERCC1* methylation, however, remained unaltered after exposure to either $PM_{2·5}$ or black carbon across the entire pregnancy. We could therefore infer that, although the ERCC1-4 complex can initiate 5′ cleavage, the complex might lose stability. Evidence suggests that both proteins are essential to maintain the optimal functioning of the NER pathway and loss of one can result in a loss in the potency of DNA repair.[30]

The tumour suppressor gene *p53* has several important roles in cell-cycle regulation, DNA recombination, development, and the induction of apoptosis.[32] We observed increased methylation of the *p53* promoter with higher $PM_{2·5}$ exposure during the entire pregnancy. In a study by Zhou and colleagues,[33] human bronchial epithelial cells were exposed to $PM_{2·5}$, and the authors reported hypermethylation in the promoter region of *p53* after 10 days of exposure.

The *DAPK1* tumour suppressor gene encodes for a calcium or calmodulin-dependent serine-threonine kinase and is involved in apoptosis. The degree of methylation in the promoter region of *DAPK1* was inversely associated with $PM_{2·5}$ exposure during the entire pregnancy, suggesting increased gene expression in response to pollutant exposure.[34] We could therefore postulate that *DAPK1* could be activated as a proapoptotic mediator by $PM_{2·5}$ pollution. This activation might, in turn, result in an increased amount of apoptotic cell death. In-vitro studies have shown that cells exposed to $PM_{2·5}$ have an increased rate of apoptosis, both via the TNF-α (ie, *DAPK1* mediated) and mitochondrially activated pathway.[35] The mechanism seems to be that as the degree of DNA damage becomes too extensive due to the suboptimal functioning of repair mechanisms, cells are redirected to initiate apoptosis to cope with this damage.

Our findings suggest that air pollution exposure during the first and second trimesters of pregnancy mainly affected tumour suppressor genes, whereas exposure later in the pregnancy affected the genes of the BER pathway. For the NER pathway, exposure across all trimesters of the pregnancy affected the *ERCC4* gene. The mutation rate marker was mainly affected by late trimester (second and third) exposure to black carbon air pollution. The NER pathway is involved in repairing DNA damage from carcinogenic compounds.[8] Our findings might indicate that the cleavage component of this pathway—ie, *ERCC4*—is susceptible to both early and late gestation exposure to air pollution exposure, whereas the BER pathway-associated genes—ie, *APEX1* and *OGG1*—are affected by late pregnancy air pollution

exposure. As mentioned earlier, the BER pathway is capable of repairing DNA damage induced by reactive oxygen species.[6] Taking these elements into account, it seems logical that the *Alu* mutation rate is associated with late gestation air pollution exposure. We could postulate that in early pregnancy, DNA damage repair is still efficient. However, when the DNA repair genes become more highly methylated, the efficiency of repair is reduced, which might contribute to an increased DNA mutation rate. This notion is in line with observations in children from the metropolitan area of Mexico City, where particulate air pollution was associated with increased methylation of DNA repair genes, as well as elevated oxidative DNA damage.[25]

We acknowledge that this study has some limitations, the first of which was the use of a single placental biopsy for each participant. However, we assessed the amount of variation within the same placenta, and although the investigated genes had a low level of methylation overall, placental intravariability was limited and ranged from 1·37% to 12·15% between biopsies originating from the same placentas. A second limitation was the variability in cell count, composition of placental tissue (which consists of syncytiothrophoblasts, cytotrophoblasts, mesenchymal cells, Hofbauer cells, and fibroblasts), and population variability. Although the composition of the biopsies could affect our findings, we used a standardised sampling method to take the biopsies at a fixed location. Furthermore, the study population is representative of the segment of the Belgian population in the pregnancy period or reproductive phase of life, suggesting that the our findings are generalisable (appendix).[36] A third limitation is the fact that only the DNA methylation of promoter regions was investigated. Although the selected regions, which are transcriptionally important, showed differences in methylation with increasing exposure to $PM_{2·5}$ and black carbon, we cannot guarantee that the same effects will be observed in levels of gene or protein expression. However, DNA methylation can be regarded as a buffer to regulate and stabilise the effects of transcription factor, ensuring long-term effects, whereas gene expression alterations tend to be more transient.[37] Fourth, the pollutant exposure results of the study are based solely on maternal residential address. It is therefore possible that the study had a degree of exposure misclassification because other exposure sources that contribute to the personal exposure, such as exposure at work, during a commute, and elsewhere, were not accounted for. However, we have found that our modelled estimates of long-term residential black carbon and $PM_{2·5}$ exposure were positively associated with internal black carbon load measured in urine,[38] which shows the ability of our model to reflect individual exposure. Finally, single measurements of methylation could be regarded as a limitation due to measurement variation. Nonetheless, we investigated the reproducibility of our assays in duplicate in a subset of 14 participants. We identified significant correlations for each of the assays.

Overall, our findings suggest that in-utero exposure to particulate matter is associated with an elevated placental mutation rate, as established from the *Alu* mutation rate. This change occurred in concert with epigenetic alterations in key DNA repair and tumour suppressor genes in the placenta. Previous research has suggested that genetic instability during tumorigenesis can be directly attributed to deficiencies in DNA repair.[25] Furthermore, the inactivation of DNA repair genes can be seen as an important hallmark of cancer initiation due to the resulting genomic instability.[25] Therefore, we suggest that our findings might represent some of the earliest effects of exposure to air pollutants in the process of carcinogenesis. Lifetime cancer risk is known to increase when exposure to carcinogens occurs early in life. Given that some methylation changes induced by air pollution persist from birth to childhood,[39] as also seen with methylation changes in DNA repair genes in children,[12] it is possible that the changes we detected could persist from this in-utero exposure to air pollution into later life. Therefore, it is essential that future studies investigate whether these epigenetic changes persist and have any role in the development of carcinogenic insults later in life.

Contributors

TSN is the coordinator of the ENVIRONAGE birth cohort and designed the current study with KYN, NDS, and VB. KYN and LT measured the samples. WL and CV modelled the air pollution exposures. KYN did the statistical analysis, with guidance provided by TSN, NDS, and BGJ. Data interpretation was done by TSN, VB, and KYN. KYN and NDS produced the first draft of the manuscript. All authors critically reviewed and approved the manuscript.

Declaration of interests

We declare no competing interests.

Acknowledgments

The work is supported by the European Research Council (ERC-2012-StG.310898 and ERC-2011-StG. 282413) and by the Flemish Scientific Fund (FWO,G073315N/G082317N). We thank A Moors for coordinating the studies at the maternity ward, as well as all the midwives from the maternity ward of the East-Limburg Hospital in Genk.

References

1 Straif K, Cohen A, Samet J, eds. Air pollution and cancer. IARC scientific publication no. 161. Lyon: International Agency for Research on Cancer, 2013.

2 Anenberg SC, Schwartz J, Shindell D, et al. Global air quality and health co-benefits of mitigating near-term climate change through methane and black carbon emission controls. *Environ Health Perspect* 2012; **120:** 831–39.

3 Wick P, Malek A, Manser P, et al. Barrier capacity of human placenta for nanosized materials. *Environ Health Perspect* 2010; **118:** 432–36.

4 Zilfou JT, Lowe SW. Tumor suppressive functions of p53. *Cold Spring Harb Perspect Biol* 2009; **1:** a001883.

5 Bialik S, Kimchi A. The death-associated protein kinases: structure, function, and beyond. *Annu Rev Biochem* 2006; **75:** 189–210.

6 Kim YJ, Wilson DM 3rd. Overview of base excision repair biochemistry. *Curr Mol Pharmacol* 2012; **5:** 3–13.

7 Krokan HE, Bjoras M. Base excision repair. *Cold Spring Harb Perspect Biol* 2013; **5:** a012583.

8 Leibeling D, Laspe P, Emmert S. Nucleotide excision repair and cancer. *J Mol Histol* 2006; **37:** 225–38.

9 Wallace SS, Murphy DL, Sweasy JB. Base excision repair and cancer. *Cancer Lett* 2012; **327:** 73–89.

10 Janssen BG, Madlhoum N, Gyselaers W, et al. Cohort profile: the ENVIRonmental influence ON early AGEing (ENVIRONAGE): a birth cohort study. *Int J Epidemiol* 2017.

11 Speir ML, Zweig AS, Rosenbloom KR, et al. The UCSC Genome Browser database: 2016 update. *Nucleic Acids Res* 2016; **44:** D717–25.

12 Alvarado-Cruz I, Sanchez-Guerra M, Hernandez-Cadena L, et al. Increased methylation of repetitive elements and DNA repair genes is associated with higher DNA oxidation in children in an urbanized, industrial environment. *Mutat Res* 2017; **813:** 27–36.

13 Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 2004; **32:** e38.

14 Yang AS, Gonzalgo ML, Zingg JM, Millar RP, Buckley JD, Jones PA. The rate of CpG mutation in Alu repetitive elements within the p53 tumor suppressor gene in the primate germline. *J Mol Biol* 1996; **258:** 240–50.

15 Janssen S, Dumont G, Fierens F, Mensink C. Spatial interpolation of air pollution measurements using CORINE land cover data. *Atmos Environ* 2008; **42:** 4884–903.

16 Lefebvre W, Vercauteren J, Schrooten L, et al. Validation of the MIMOSA-AURORA-IFDM model chain for policy support: modeling concentrations of elemental carbon in Flanders. *Atmos Environ* 2011; **45:** 6705–13.

17 Lefebvre W, Degraeuwe B, Beckx C, et al. Presentation and evaluation of an integrated model chain to respond to traffic- and health-related policy questions. *Environ Model Soft* 2013; **40:** 160–70.

18 Maiheu B, Veldeman N, Viaene P, et al. Identifying the best available large-scale concentration maps for air quality in Belgium. 2013. https://www.milieurapport.be/publicaties/2013/bepaling-van-de-best-beschikbare-grootschalige-concentratiekaarten-luchtkwaliteit-voor-belgie (in Dutch; accessed May 20, 2017).

19 Beelen R, Raaschou-Nielsen O, Stafoggia M, et al. Effects of long-term exposure to air pollution on natural-cause mortality: an analysis of 22 European cohorts within the multicentre ESCAPE project. *Lancet* 2014; **383:** 785–95.

20 European Parliament, Council of the European Union. Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe. Brussels: European Union, 2008.

21 Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994; **54:** 4855–78.

22 Tsai AG, Lu H, Raghavan SC, Muschen M, Hsieh CL, Lieber MR. Human chromosomal translocations at CpG sites and a theoretical basis for their lineage and stage specificity. *Cell* 2008; **135:** 1130–42.

23 Perera F, Hemminki K, Jedrychowski W, et al. In utero DNA damage from environmental pollution is associated with somatic gene mutation in newborns. *Cancer Epidemiol Biomarkers Prev* 2002; **11:** 1134–37.

24 Zhang W, Bouffard GG, Wallace SS, Bond JP, NISC Comparative Sequencing Program. Estimation of DNA sequence context-dependent mutation rates using primate genomic sequences. *J Mol Evol* 2007; **65:** 207–14.

25 Lahtz C, Pfeifer GP. Epigenetic changes of DNA repair genes in cancer. *J Mol Cell Biol* 2011; **3:** 51–58.

26 Tell G, Quadrifoglio F, Tiribelli C, Kelley MR. The many functions of APE1/Ref-1: not only a DNA repair enzyme. *Antioxid Redox Signal* 2009; **11:** 601–20.

27 Xie Y, Yang H, Cunanan C, et al. Deficiencies in mouse *Myh* and *Ogg1* result in tumor predisposition and G to T mutations in codon 12 of the *K-ras* oncogene in lung tumors. *Cancer Res* 2004; **64:** 3096–102.

28 Miller-Pinsler L, Pinto DJ, Wells PG. Oxidative DNA damage in the in utero initiation of postnatal neurodevelopmental deficits by normal fetal and ethanol-enhanced oxidative stress in oxoguanine glycosylase 1 knockout mice. *Free Radic Biol Med* 2015; **78:** 23–29.

29 Harper JW, Elledge SJ. The DNA damage response: ten years after. *Mol Cell* 2007; **28:** 739–45.

30 de Laat WL, Jaspers NG, Hoeijmakers JH. Molecular mechanism of nucleotide excision repair. *Genes Dev* 1999; **13:** 768–85.

31 Wood RD. Nucleotide excision repair in mammalian cells. *J Biol Chem* 1997; **272:** 23465–68.

32 Friedberg EC. How nucleotide excision repair protects against cancer. *Nat Rev Cancer* 2001; **1:** 22–33.

33 Zhou W, Tian D, He J, et al. Repeated PM2.5 exposure inhibits BEAS-2B cell P53 expression through ROS-Akt-DNMT3B pathway-mediated promoter hypermethylation. *Oncotarget* 2016; **7:** 20691–703.

34 Lim DHK, Maher ER. DNA methylation: a form of epigenetic control of gene expression. *Obstetrician Gynaecologist* 2010; **12:** 37–42.

35 Dagher Z, Garcon G, Billet S, et al. Activation of different pathways of apoptosis by air pollution particulate matter (PM2.5) in human epithelial lung cells (L132) in culture. *Toxicology* 2006; **225:** 12–24.

36 Cox B, Martens E, Nemery B, Vangronsveld J, Nawrot TS. Impact of a stepwise introduction of smoke-free legislation on the rate of preterm births: analysis of routinely collected birth data. *BMJ* 2013; **346:** f441.

37 Smith ZD, Meissner A. DNA methylation: roles in mammalian development. *Nat Rev Genet* 2013; **14:** 204–20.

38 Saenen ND, Bove H, Steuwe C, et al. Children's urinary environmental carbon load. A novel marker reflecting residential ambient air pollution exposure? *Am J Respir Crit Care Med* 2017; **196:** 873–81.

39 Gruzieva O, Xu CJ, Breton CV, et al. Epigenome-wide meta-analysis of methylation in children related to prenatal NO_2 air pollution exposure. *Environ Health Perspect* 2017; **125:** 104–10.