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BC	Black Carbon
BMI	Body Mass Index
CI	Confidence Interval
CpG	Cytosine-phosphate-Guanosine
FWO	Research Foundation-Flanders
IFDM	Immision Frequency Distribution Model
MANOE	MAternal Nutrition and Offspring Epigenome
MD	Missing Data
PC	Principle Component
PCA	Principle Component Analysis
PM	Particulate Matter
PM_{10}	Particulate Matter with an aerodynamic diameter ≤ 10 µm
$PM_{2.5}$	Particulate Matter with an aerodynamic diameter ≤ 2.5 µm
VITO	Flemish Institute of Technological Research
WFL	Weight-For-Length

The prevalence of childhood obesity is rapidly increasing. Therefore, gaining more information on the role of environmental parameters is key. With overexpression of leptin (encoded by *LEP*) in obesity, *LEP* methylation might be altered by environmental exposures. This study aims to assess effects of ambient air pollution and nearby greenness on obesity-related growth and *LEP* methylation in early childhood. We monitored 120 mother-child pairs from conception until the age of five. Buccal swabs and anthropometric measurements of the children were taken at six months, one year, and five years old. Buccal DNA was extracted to determine *LEP* methylation levels. Estimates of air pollution and nearby greenness were calculated using high-resolution models. Effects of air pollution and nearby greenness on growth or *LEP* methylation were investigated using linear mixed effects models. Positive associations were shown for air pollution between conception and age one on impedance in six-month-olds and one-year-olds in the crude model. PM with aerodynamic diameter ≤10 (PM_{10}) and ≤2.5 μm ($PM_{2.5}$) positively associated with waist-hip-ratio and waist circumference at age five in the fully adjusted model. In early childhood, closest distance to forest negatively, and urban green and forest positively associated with weight-for-length, body mass index, and fat percentage in five-year-olds in the fully adjusted model. No significant associations for noise, and walkability on growth were seen. Negative associations were shown for smaller green clusters and positive associations for greater green clusters on *LEP* methylation in one-year-olds. For forest distance, walkability, noise, or all green on *LEP* methylation, no significant associations were found. Evidence is provided that ambient air pollution might have a significant effect on impedance and waist-hip-ratio, suggesting an increased risk of childhood obesity. Based on *LEP* methylation, greater green clusters might associate with a decreased risk of childhood obesity, while smaller green clusters showed the opposite.

Keywords: Childhood obesity; Environmental health; Ambient air pollution; Green space; Leptin; DNA methylation.

Introduction

Worldwide, the prevalence of childhood obesity increased eightfold between 1975 and 2016 (1,2). In 2018 in Belgium, 5.8 % of the two- to seventeen-year-olds, and 11.7 % of the two- to four-year-olds were obese (3). The pathophysiology of obesity is complex with interactions between genetic, biological, and environmental factors, such as nutrition and physical activity, lying at its basis (4–6).

Environmental exposures, like air pollution and accessibility to greenness, starting from conception might contribute to the development of childhood obesity (7–19). Green spaces can have beneficial effects on health by creating opportunities, e.g. encouraging physical activity, and it is thought to reduce harmful exposure to heat, traffic and air pollution (20,21). On the other hand, studies that investigated available greenness, and childhood obesity showed contradicting results (15–17). These contradictions might be explained by mediation of air pollution.

Particulate matter (PM) is the key indicator of air pollution (7,22–25). $PM_{2.5}$, PM_{10} and NO_2 have shown associations with an increased risk of childhood obesity (19,26–30). Air pollution induces oxidative stress, and results in immediate, but also alterations in later immune responses (10,22,23,31). While the underlying mechanisms have not been entirely clarified, several studies found a link with the human epigenome (32).

DNA methylation in young children can affect development and physiology their whole life (33). Changes in methylation caused by environmental stimuli can play a role in their pathologic effects (24). Studies have related several air pollution components, such as PM, ozone and nitric oxides, to lower global methylation levels (34–36). These changes are suggested to be able to influence long-term effects on gene expression and thereby cell function (37).

Leptin (encoded by *LEP*) is part of the hypothalamic leptin-melanocortin regulating pathway, in which single gene mutations can result in obesity (6,38–41). Leptin, mainly produced by white adipose tissue, is involved in body weight and height (6,42–44). Leptin levels are proportional to body fat mass, but in obesity, the expected effects from the high levels are abnormal, called leptin resistance (44–47). It has been shown that air pollution correlates to altered DNA methylation, including in the *LEP* promotor (48).

Multiple studies have shown a link between *LEP* methylation and obesity-related growth (49–52). *LEP* methylation from buccal swabs results mainly in the DNA profile of the oral mucosa. This collection method is minimally invasive and easy to perform in young children. The role of leptin in the oral mucosa is still unclear (53). However, it has been shown before that DNA methylation of the oral mucosa positively correlates to diverse tissues (54–56).

This particular study aims to assess the impact of pre- and postnatal air pollution and green exposure on obesity-related growth parameters and *LEP* methylation. We hypothesise that air pollution exposure

leads to increased weight-for-length (WFL) and body mass index (BMI), and reduced *LEP* methylation in early childhood. Therefore, we expect an increased risk of childhood obesity with more exposure to air pollution and lower availability to green spaces.

Materials and Methods

Study population

This study is a follow-up of children born in the Maternal Nutrition and Offspring's Epigenome (MANOE) cohort, described before by Pauwels *et al.* (57–61) and Daneels *et al.* (62). Written informed consent was obtained from all mothers upon recruitment. This study was conducted according to the guidelines laid down in the Declaration of Helsinki. The study protocol, including all procedures involving human subjects, was approved by the UZ Leuven-Committee for Medical Ethics (S61606, ML7975).

Anthropometric measurements in children

Children from the MANOE cohort were followed up at birth, six months, one year, and five years old. Included children were born between February 2013 and March 2016. Using a general questionnaire, information on health status and lifestyle were collected. At each visit, length, weight, and bio-electrical impedance (Bodystat 1500®; not measured at birth) were determined. Based on sex and age, z-scores were calculated for weight, BMI, and WFL using the Belgian 2004 Growth Reference Chart (63). WFL z-score was used to define the following categories: underweight (<5 %), normal weight (5-85 %), and overweight (>85 %). At age five, waist and hip circumference were measured. Waist-hip-ratio was determined by dividing waist by hip circumference. Fat-free mass was calculated using the formula of Schaefer *et al.* (64) for five-year-olds based on the bio-electrical impedance measurement. *Fat Percentage = 1 – (Fat-free mass/Body Weight).* Fat percentages were logit-transformed and percentages of 0 or below, received -2.5 as logit-transformed value.

Sample collection and DNA methylation analysis

Oral mucosal epithelial cells were collected at six months old, one year old, and five years old as described by Pauwels *et al.* (60,61). DNA extraction and bisulfite conversion were performed as previously described (59–61). Bisulfite converted DNA was amplified using commercially available *LEP* primers (#PM00129724 PyroMark CpG Assays; Qiagen) as described earlier (59,61). DNA methylation of four CpG sites located in the promotor region was analysed using pyrosequencing in the Pyromark Q48 Autoprep (Qiagen; version 4.2.1) according to the manufacturer's instructions. Methylation percentages were logit-transformed with -5 as value for DNA methylation levels of 0%. Variabilities between PCR's and pyrosequencing runs are noted in **Appendix A**.

Leptin concentration measurement

Leptin concentrations were determined in plasma samples, collected at five years old. Details are described in **Appendix O**.

Modelled air pollution

Air pollution exposure data was obtained from the Belgian Interregional Environment Agency. Home addresses of participants were geocoded and address changes were considered. Data of exposure to black carbon (BC), PM_{10}, $PM_{2.5}$, NO_2, and O_3 was obtained for 2012 to 2021. Based on annual and daily (only for 2021) averages of exposure, periodical daily averages (for in utero, birth to six months old, six months to one year old, and one to five years old) were calculated by the summation of the multiplications of the number of days in the year times the air pollution factor daily average in that year for all calendar years in that period. Exposure data before the child turned 3.5 years was derived from the RIO-Immision Frequency Distribution model (IFDM), while data after the child turned 3.5 years came from the RIO-IFDM-Operational Street Pollution Model (ATMO-Street (65)), which also contains a street canyon module. For the missing BC in 2016 and ozone in all years in the ATMO-Street model, the RIO-IFDM model was used. For Belgian data, the RIO-IFDM explained >70 % (R^2 >0.70) of the temporal and > 45 % (> 80 % for NO_2) (R^2 > 0.45; > 0.80 for NO_2) of the spatial variability of the investigated air pollution factors (66). No validation of BC data was available. For one address air pollution could not be estimated, but the impact is thought to be minimal because the participant moved after the first five days of pregnancy.

Residential greenness

Residential addresses were geocoded using BeSTAddress (FOD BOSA, version 2020). High-resolution land cover models were used to determine different environmental parameters for the period of 2012 to 2021. Environmental data was obtained as described by Bijnens *et al.* (20) and Verheyen *et al.* (67). For six-month-olds and one-year-olds, the land-use map of Flanders of 2015 were used, while for five-year-olds the land-use maps of Flanders of 2018 were chosen. The following environmental parameters were assessed: green space (contains all green excluding agricultural green), high green space (contains vegetation higher than 3m), agricultural green space, all green (contains low, high and agricultural green). These green parameters were derived from high-resolution land cover data (1 m^2, converted to 10 x 10 m resolution for calculations; called the Green Map of Flanders from the Agency for Geographic Information Flanders). The indicators were calculated for different radii from the home address (50 m, 100 m, 300 m, 500 m, 1,000 m, and 2,000 m) and expressed as a percentage of area coverage.

Further, we assessed noise exposure (MIRA/UGent, 2015 (68), 2018 (69)), walkability score (measure for walker friendliness; “Vlaams Instituut Gezond Leven, 2018” (70); expressed as a z-score), and closest distance to forest (non-agricultural green space > 0.5 ha and tree canopy ≥ 30 %). Noise exposure

is expressed as L_{den}, being average sound level over a 24-hour period with evening and night-time hours added penalties of 5 and 10 dB, respectively.

Additionally, we used green indicators that represent the presence of several sizes of green clusters at certain distances from the home address based on availability and accessibility (71): green in neighbourhood (>20 a at <400 m), green in district (>10 ha at <800 m), small urban green (>30 ha at <1 600 m), urban green (>60 ha at <3,200 m), and urban forest (>200 ha at <5,000 m) with distances calculated by road. Environmental parameters, except for walkability, could not be determined for one individual living outside of the study area of Flanders (Region of Belgium).

Statistics

Data analysis and processing was performed in R (version 4.1.3; R Core Team 2022) (72). All statistical tests were two-sided, and $p<0.05$ was considered significant. Using boxplots and Estimated Student's Deviate tests at all time points and for the longitudinal data, outliers were detected and normality was checked using Shapiro-tests. Potential correlations were explored using correlation tests (Pearson's method for normal data, Spearman's method for not-normal distributed data) and principle component analysis (PCA) (data not shown).

Differences between sexes or over time were assessed using one-way repeated measures ANOVA. Differences were further explored using paired (over time) or unpaired (between sexes) t-tests (for normal data) and Wilcoxon signed rank (over time) or Wilcoxon rank sum (between sexes) (for not-normal data). Variances were checked.

Longitudinal impact of environmental parameters and air pollution on growth and *LEP* methylation were investigated using linear mixed effects models with subject identifier as random effect. For fat percentage, hip and waist circumference, and waist-hip-ratio, linear regression was used. Four a priori chosen models were used. The first model was unadjusted. Next, we controlled for age (years) at the moment of measurement and sex in the second model. For the models with environmental factors, in the second model, also birth weight (kg) and birth length (cm) were controlled for. Next, the model was further adjusted for maternal factors, namely maternal age at conception (years), education, occupation, all at recruitment, maternal pre-pregnancy BMI, and parity (model 3). The category of highest education level at time of recruitment was used as maternal education: High school degree, 7^{th} year of high school, Bachelor's degree, Advanced bachelor, Master's degree, Advanced master, or PhD. For maternal occupation, the following categories were used: not currently employed, employed in education, health care, research/science, social welfare, administration/logistics, legal sector, trade/catering, banking/insurance/financial services, or other. In the fourth model, we further adjusted for pregnancy-related factors with potential influence on foetal growth: active smoking during pregnancy (yes/no), folate intake (µg/day), alcohol consumption during pregnancy (yes/no), maternal gestational weight gain (kg), mode of delivery (vaginal/caesarean/instrumental), season of conception, gestational age at birth

(weeks). Folate intake was determined on a 7-day estimated dietary record at twelve weeks pregnancy. Restricted maximum likelihood was used to fit the linear mixed effects models, and unpaired t-tests used Satterthwaite's method. Models were created with exposure measurements earlier or at the same time as the outcome measurements to assure a potential interference.

Model 1: Growth/DNA Methylation ~ Environmental parameter

Model 2: Growth/DNA Methylation ~ Environmental parameter + Age + Sex (+ Birth Length + Birth Weight)

Model 3: Growth/DNA Methylation ~ Environmental parameter + Age + Sex (+ Birth Length + Birth Weight) + Maternal age + Maternal education + Maternal occupation + Maternal pre-pregnancy BMI + Parity

Model 4: Growth/DNA Methylation ~ Environmental parameter + Age + Sex (+ Birth Length + Birth Weight) + Maternal age + Maternal education + Maternal occupation + Maternal pre-pregnancy BMI + Parity + Smoking + Folate intake + Alcohol use + Maternal gestational weight gain + Delivery Mode + Gestational age at birth + Season of conception

Due to the high correlation **(Appendix B)** between air pollution parameters, dimensions were reduced by first assessing PM_{10}. To reduce the dimensions further, first z-score of BMI and WFL, waist-hip-ratio, impedance, and logit-transformed fat percentage were assessed. When a significant association of environmental parameters on growth or DNA methylation was found, the associations with specific periods of growth or DNA methylation was investigated. If there were significant results, associations with other environmental factors were investigated. In a next step, other relating growth factors were investigated.

To reduce dimensions of parameters of greenness, we first assessed all green, greenness factors, forest distance, walkability score, and noise. Principle component (PC) analyses (PCA) were used to further reduce dimensions for all green and the greenness factors **(Appendix C)**. The two main PCs of all green and greenness factors were used. Dimension reduction on growth and DNA methylation was identical as mentioned earlier.

Finally, the role of air pollution as a mediator in the associations seen for greenness on growth and *LEP* methylation was assessed and the role of LEP methylation as mediator in the associations between air pollution and greenness factors and obesity-related growth. Mediation analyses were performed as described by Baron and Kenny (73). To show significance, bootstrapping was used with 10,000 repeats. Mediation analyses was performed on unadjusted linear models.

Results

Descriptive analysis of study characteristics

Study population

Between April 2012 and February 2017, a total of two hundred and fourteen (214) mother-infant pairs were recruited **(Figure 1)**. Drop-outs during the follow-up in different stages of the study (n=93; n=52 aged six months, n=9 aged one year, and n=32 aged five years) or children with missing buccal swab at one of the follow-up visits (n=1) were excluded. A total of 120 children, born between February 2013 and March 2016 were included in the statistical analyses. This sample size was estimated to be sufficient based on previous studies (60). During pregnancy, women also were asked to keep a food frequency questionnaire (61,74) in order to determine methyl donor intake.

Characteristics of growth

Of the 120 children included in the study, 52.5 % were boys **(Appendix D)**. Weight z-scores showed no differences between sexes. BMI averaged 17.13 ± 1.35 at six months old, 17.31 ± 1.46 at one year old, and 15.26 ± 1.25 kg/m² at five years old. Twenty percent of the children was overweight at six months old, 24.17% at one year old, and 8.33% at five years old. Girls showed a higher impedance measurement, while waist-hip-ratio was higher for boys. Maternal characteristics are available in **Appendix E**.

Characteristics of air pollution exposure

Air pollution exposure was determined during pregnancy, between birth and six months old, between six and twelve months old, and between one and five years old based on the home address of the children **(Figure 2)**. Average BC, $PM_{2.5}$, and NO_2 exposure gradually decreased with increasing age from 1.16 ± 0.26 to 0.86 ± 0.16, 13.30 ± 1.24 to 11.99 ± 0.76, and 19.75 ± 4.43 to 17.31 ± 3.37 µg/m³ between pregnancy and the period of one to five years old, respectively. PM_{10} decreased between pregnancy and the period from one to five years old from 18.98 ± 1.81 to 18.40 ± 1.21 µg/m³, while O_3 increased from 44.36 ± 2.77 to 46.57 ± 2.81 µg/m³, respectively. Both PM_{10} and O_3, exhibited periods of stability in between. In this study population, air pollution concentrations are higher than the levels recommended in the World Health Organization (WHO) guidelines from 2021 (75). Maximal recommended air pollution concentrations for PM_{10}, $PM_{2.5}$, and NO_2 are 15, 5, and 10 µg/m³, respectively.

Characteristics of green spaces, traffic-related noise and walkability

In this study, on average 35.14 ± 18.65, and 3.55 ± 7.91 % of the area within 2,000 m, and 50 m, respectively, was agricultural green **(Appendix F)**. Within 2,000 m, and 50 m, 23.67 ± 10.19, and 14.73 ± 12.21 % of the area, respectively, was of high green space. For green space, average coverage of 40.62 ± 11.83 % within 2,000 m, and 37.32 ± 15.18 % within 50 m was present. All green space was present on average for 75.76 ± 12.55 % of the area within 2,000 m and for 40.87 ± 16.52 % within 50 m. On average, participants were exposed to 59.55 ± 6.24 L_{den} of noise and the average walkability score was

2.29 ± 4.32. The closest forest was situated at on average 232.69 ± 265.80 m. For almost all children green in neighbourhood (98.33 % for six-month-olds and one-year-olds, 95.83 % for five-year-olds), urban green (96.67 % for six-month-olds and one-year-olds, 95 % for five-year-olds), and urban forest (96.67 % for the three data points) were accessible. Green in district was accessible for 78.33 %, 79.17 %, 78.33 %, and small urban green for 86.67 %, 86.67 %, 85 % of the children at six months, one year, five years old, respectively.

Environmental greenness did not change over time with some exceptions. High green space within 2,000 m and 1,000 m was higher in five-year-olds than in six-month-olds ($p<0.001$, $p<0.01$, respectively), and one-year-olds ($p<0.01$, $p<0.05$, respectively). Green space within 50 m was higher at six months than at one year old ($p<0.05$). Finally, all green within 50 m was higher at one year old ($p<0.05$) and five year old ($p<0.05$) compared to six months old (data not shown).

Characteristics of *LEP* methylation

Mean *LEP* methylation increased with increasing age from 9.23 ± 1.95 at age six months to 10.08 ± 2.23 at one year old to 11.11 ± 2.06 at age five **(Figure 3)**. Differences between sexes are discussed in **Appendix G**.

Characteristics of leptin concentrations

Leptin concentrations were on average 2.34 ± 1.74 ng/ml (range: 0.026-9.168 ng/ml) (girls: 2.36 ± 1.26 (range: 0.051-6.131 ng/ml); boys: 2.32 ± 2.18 ng/ml (range: 0.026-9.168 ng/ml)). The correlation coefficient between leptin concentrations and logit-transformed mean *LEP* methylation was -0.054 (for girls: -0.065; for boys: 0.070), however, the correlations were not significant (**Appendix O)**.

Effect of environmental factors on growth and *LEP* methylation

Effect of air pollution on growth

For the effect of air pollution on growth, we first looked at potential effects of PM_{10} on growth parameters **(Table 1; Appendix H)**. Significant effects of PM_{10} were found during the period of one year to five years old on waist-hip-ratio, and during pregnancy, between birth and six months, and between six months to one year old on impedance. No significant effects were found of PM_{10} on z-score of WFL, z-score of BMI, and logit-transformed fat percentage.

Next, for waist-hip-ratio, we investigated the potential effects of other air pollution factors between one to five years of age. A positive significant effect was found for $PM_{2.5}$. For that same exposure period, waist circumference was significant for both PM_{10}, and $PM_{2.5}$, but only in the fully adjusted model. Hip circumference did not associate significantly with PM_{10} or $PM_{2.5}$ during the period of one to five years old.

For the associations of PM_{10} on impedance, next, the effects were determined for specific impedance measurements. Significant associations were found for PM_{10} during pregnancy on impedance at one

year old, during birth to six months old on impedance at six months old, between six months and one year old on impedance at one year old. In a next step, it was checked whether other air pollution factors showed the same associations. During pregnancy, $PM_{2.5}$, BC, NO_2, and O_3 showed significant associations with impedance at age one. Between birth and six months of age, $PM_{2.5}$ and BC associated positively with impedance measurements at six months old. Finally, $PM_{2.5}$ and BC between the ages of six months old to one year old significantly associated with impedance at one year old. Adapted methods for effects on growth at birth with their results can be found in **Appendix I.**

Effect of air pollution on *LEP* methylation

No associations were found for PM_{10} on mean *LEP* methylation **(Appendix J)**.

Effect of green spaces, noise, and walkability on growth

For effects of forest distance, walkability, and noise on growth, significant effects were seen for forest distance at age five on z-score of WFL, z-score of BMI, and logit-transformed fat percentage, and for walkability at age five on impedance **(Table 2; Appendix K)**. In a next step, it was investigated if the significant associations of forest distance and walkability on growth were also present for relevant periods of these growth parameters. The effects of forest distance in five-year-olds, significantly associated with z-score of WFL, z-score of BMI. Additionally, the associations between all green and greenness factors on the one hand and growth parameters on the other hand were investigated. No significant associations for all green on growth were seen.

For the associations of the principle components of greenness factors on obesity-related growth parameters, several significant effects were seen. PC2 at six months of age significantly associated with z-score of WFL, z-score of BMI, and waist-hip-ratio. PC2 in one-year-olds showed significant associations with z-score of BMI, and waist-hip-ratio. At age five, PC1 and PC2 associated significantly with logit-transformed fat percentage. In a next step, it was investigated which specific greenness factors showed significant associations with growth. All significant associations were for growth at age five and with urban green or urban forest, except green in the neighbourhood at five years old that significantly associated with fat percentage in five-year-olds. Urban green and urban forest at six months old positively associated with five year old z-score of WFL and BMI, at one years old with z-score of BMI, and at five years old with fat percentage. Based on the significant associations on BMI or WFL z-scores, it can be investigated if these associations are present for weight z-score or length as well. Significance was shown for urban green and forest at six months old and one year old on z-score of weight in five-year-olds.

Effect of green spaces, noise, and walkability on *LEP* methylation

When investigating the effect of forest distance, walkability, noise, or all green on mean *LEP* methylation, no significant associations were found **(Table 3; Appendix L)**. For the associations of PCs

of greenness factors with mean *LEP* methylation, significance was found for PC2 at six months old, at one year old, and at five years old.

When effects of specific greenness factors on *LEP* methylation are investigated, only methylation at one year old showed significant associations with greenness factors. Green in district and small urban green at six months old and one year old negatively associated, while urban forest positively associated with one year old *LEP* methylation.

Mediation of air pollution on green spaces, noise, and walkability

For the twenty-two significant associations of green spaces, noise, and walkability on obesity-related growth or *LEP* methylation, it was investigated whether these might be mediated by air pollution **(Appendix M)**. Urban forest at six months, one year and five years old was mediated by PM_{10} for its associations on WFL z-score, BMI z-score, and fat percentage at age five between 0.40 % (95% CI (-1,219.76;1.220,56)) and 3.55 % (95% CI (-216,83;223,92)).

Mediation of *LEP* methylation on air pollution and green spaces, noise and walkability

For the thirty-one significant associations of air pollution, green spaces, noise, and walkability on obesity-related growth, the role of *LEP* methylation as potential mediator was assessed **(Appendix N)**. Only on the association of urban forest on fat percentage, *LEP* methylation (all at the age of five) mediated for 7.09 % (95% CI (-1.219,56; 1.233,73)).

Discussion

During the critical time window of the first 1,000 days of life, these children were exposed to high concentrations of air pollution, that reduced throughout follow-up. That reduction can be explained by actions taken to improve the quality of air. The government took actions such as adapting policies for industrial exhaust, for transport and mobility, and for agricultural sources (76). It is also expected that the measures taken regarding the SARS-CoV-19 virus play a role in this improvement of air quality for the children with follow-up in or after the pandemic (77,78).

By comparing with the most recent (2021) WHO guidelines for air pollution factors (75), average concentrations exceed these thresholds. $PM_{2.5}$ concentrations were even double of the recommended maximum annual mean. This study provides evidence supporting that air pollution does affect growth and the associations seen with impedance and waist-hip-ratio, suggest that air pollution might increase the risk of obesity. These findings are in line with what has been shown in literature (24,79–86). This suggests that the European guidelines for air pollution are not strict enough and should be lowered to protect pregnant women and young children and to contribute to the prevention of childhood obesity.

In contrast to air pollution, green spaces, noise, and walkability were relatively stable over time. Only associations were shown with growth at age five, suggesting that a latency period might be present. Notably, the seen associations all tend to associate more presence of nearby green with increased

obesity-related growth parameters, suggesting more nearby green might associate with an increased risk of childhood obesity. Previous studies showed negative associations between nearby green and weight and BMI (15,16,87–90). With the hypothesis that green space gives rise to opportunities such as physical activity (21), associations between more green and increased obesity-related growth parameters were not expected. A potential explanation is that our study population was younger, and therefore, more dependent on their parents. Other studies have shown that due to increasing autonomy and lifestyle changes, green spaces affect weight status more clearly with increasing age (91). We also saw more associations for greenness factors at age five, such as forest distance and green in the neighbourhood. However, another study with children aged four to eight found no associations between greenness and BMI (17). Additionally, nearly our entire population had accessibility and availability of greenness, which might explain these contradicting results.

Additionally, we also studied *LEP* methylation as a potential actor in the interactions between the environment and obesity-related growth, because leptin is commonly overexpressed in obesity. Mean *LEP* methylation increased with increasing age. Kochmanski *et al.* (92) also showed an increase in mean *LEP* methylation levels from one to two years old to three to five years old, but they assessed a different amplicon. Dunstan *et al.* (93) used the same amplicon as this study, but in an older population. They noted mean methylation levels of 18.92% in ten- to fifteen-year-olds, suggesting that *LEP* methylation increases even more during childhood. For *LEP* methylation, it has been shown that it decreases leptin expression (94,95). Thereby, it is suggested that the increase in mean *LEP* methylation with increasing age leads to a decrease in leptin expression.

Several plausible mechanisms have been proposed by which air pollution might affect childhood obesity, including epigenetic modulation (96). This study showed no associations for PM_{10} on mean *LEP* methylation. While no studies were found linking leptin levels in children to air pollution, it has been shown that serum leptin increases with increasing near-roadway air pollution in obese young adults (97). Therefore, it remains a possibility that also in children air pollution affects leptin expression possibly not regulated at methylation level. Notably, changes in DNA methylation in buccal tissue might not correlate to changes in adipose tissue, which is the main producer of leptin (43,44,98).

The results of the assessment of associations between green spaces, noise, or walkability and *LEP* methylation levels suggest that the size of, or distance to green clusters determines the direction of the association. Larger study populations might be needed to show a significant effect of green in neighbourhood on mean *LEP* methylation. Because DNA methylation in promotor regions usually represses gene expression (24,34,94), it can be suggested that presence of smaller green clusters, might be associated with increased leptin expression. Thereby, presence of smaller green clusters, might associate with an increased risk of childhood obesity, while the opposite is possible for greater green clusters, farther away.

Air pollution only mediated in very low amounts (all below 4%) in the relationship of greenness on growth. No mediation of air pollution was seen on *LEP* methylation. Since, we expect the effects of greenness to be indirect, this suggests that they might be mediated by other air pollution factors or factors unrelated to air pollution. The proportion mediated was not significant in our dataset, which could be due to a lack of statistical power, because our sample size is not large enough to show these mediations.

Strengths and limitations

The strengths of this study are the usage of longitudinal data with diverse objective anthropometric measures of a substantial group of children (n =120) on four postnatal data points (birth, six months, one year, and five years old). Data was collected from the mothers starting at the first echography allowing objective data collection. Different environmental parameters were calculated objectively using the available Belgian organisations. Five different air pollution components were investigated and multiple parameters of green were assessed with several radii of greenness and availability and accessibility to different green clusters. To assess potential interactions, we used four nested models that control each time for more potential important variables. Finally, this study investigates both growth and the epigenetic context at the *LEP* gene.

A limitation of this study is that air pollution exposure is only assessed at home, while the study participants also spend significant amounts of time at other locations such as work, day care, … Daily air pollution exposure measurements would allow to assess shorter periods, such as trimester-specific exposure. For this study, we assessed DNA methylation deriving from buccal swabs, while DNA methylation can differ between cell types (98). Our chosen method also did not allow to differentiate between methylation and hydroxymethylation. We chose to only assess *LEP* methylation, while several other genes might play a role in obesity. To generalize findings, experiments would have to be repeated in another cohort and in other age categories of children.

Future research

With the limitations in mind, several possibilities arrive for future research. More time points can be added to investigate specific periods of high vulnerability, including trimester-specific exposure. It might also be interesting to include the quality of green, which is suggested to be involved in the reluctance of parents to allow unsupervised activities in greenness (99). For air pollution exposure, it can be interesting to question participants for average distribution at other places than home for mothers, such as work, and for children, such as day care to get a more accurate estimate of exposure to ambient air pollution. It can also be interesting to include physical activity of these pregnant women and young children as a contributor to weight status or a mediator for the effects of environmental parameters. To be able to draw more definite conclusions, leptin can also be measured in blood to confirm the increase in concentration with a decrease in methylation in buccal DNA. Because obesity is a complex disease, many other potential obesity related genes could be investigated using a similar approach.

Conclusion

This study provides evidence that ambient air pollution might have a significant effect on growth with increased impedance and waist-hip-ratio due to waist circumference. This suggests that ambient air pollution might increase the risk of childhood obesity. For more nearby green, associations suggest an increased risk of childhood obesity, however, this is probably due to other unaccounted factors. Based on *LEP* methylation, greater green clusters might associate with a decreased risk of childhood obesity, while smaller green clusters showed the opposite.

TABLES AND FIGURES

FIGURES

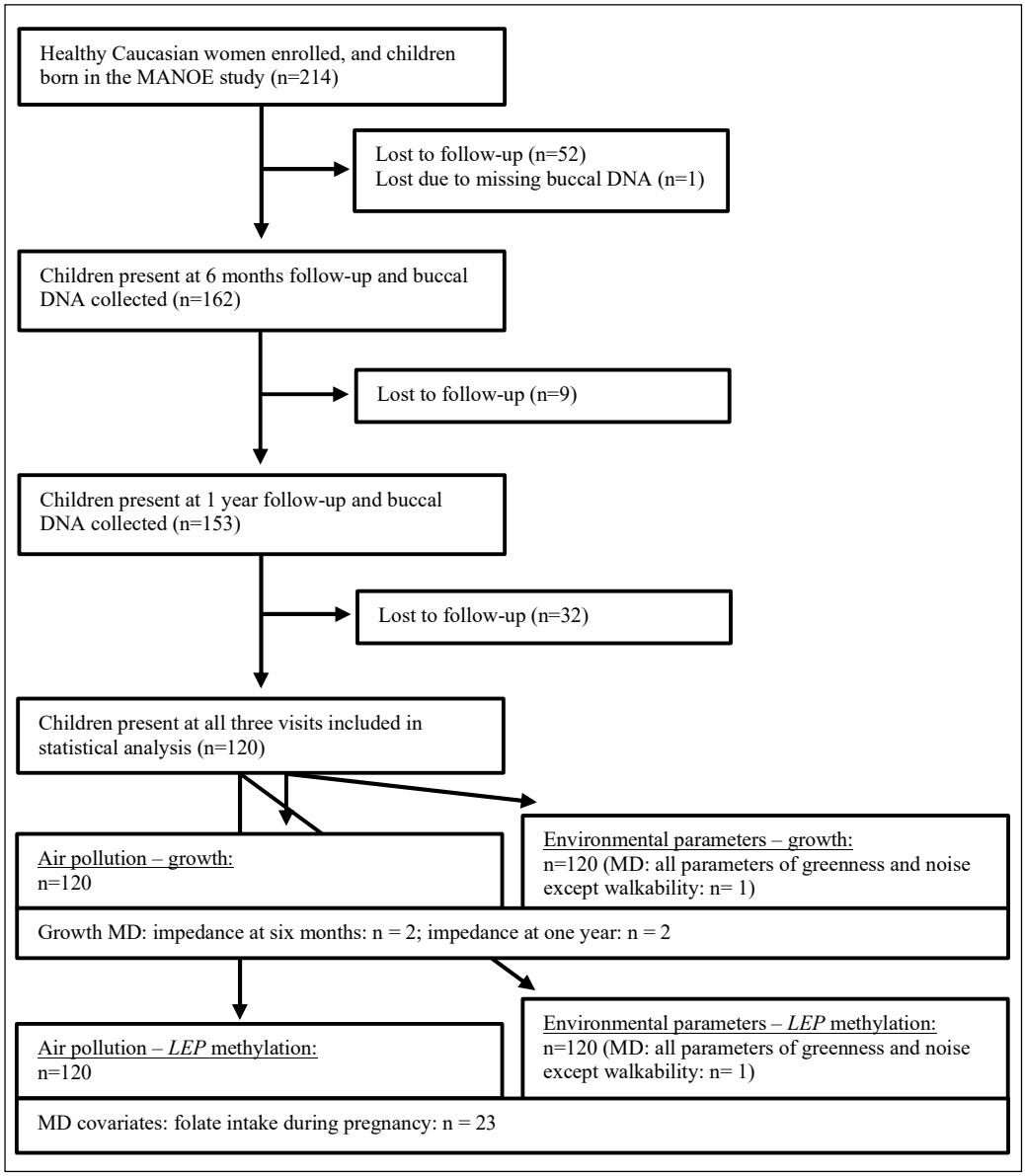


Figure 1: Flow chart of the children included in the analysis. Number of participants is shown. Finally, 120 children were included in the statistical analyses. There were missing data (MD) for impedance at six months (n=2), impedance at one year (n=2), folate intake during pregnancy (n=23), and all environmental factors except for walkability (n=1). MANOE, MAternal Nutrition and Offspring's Epigenome.

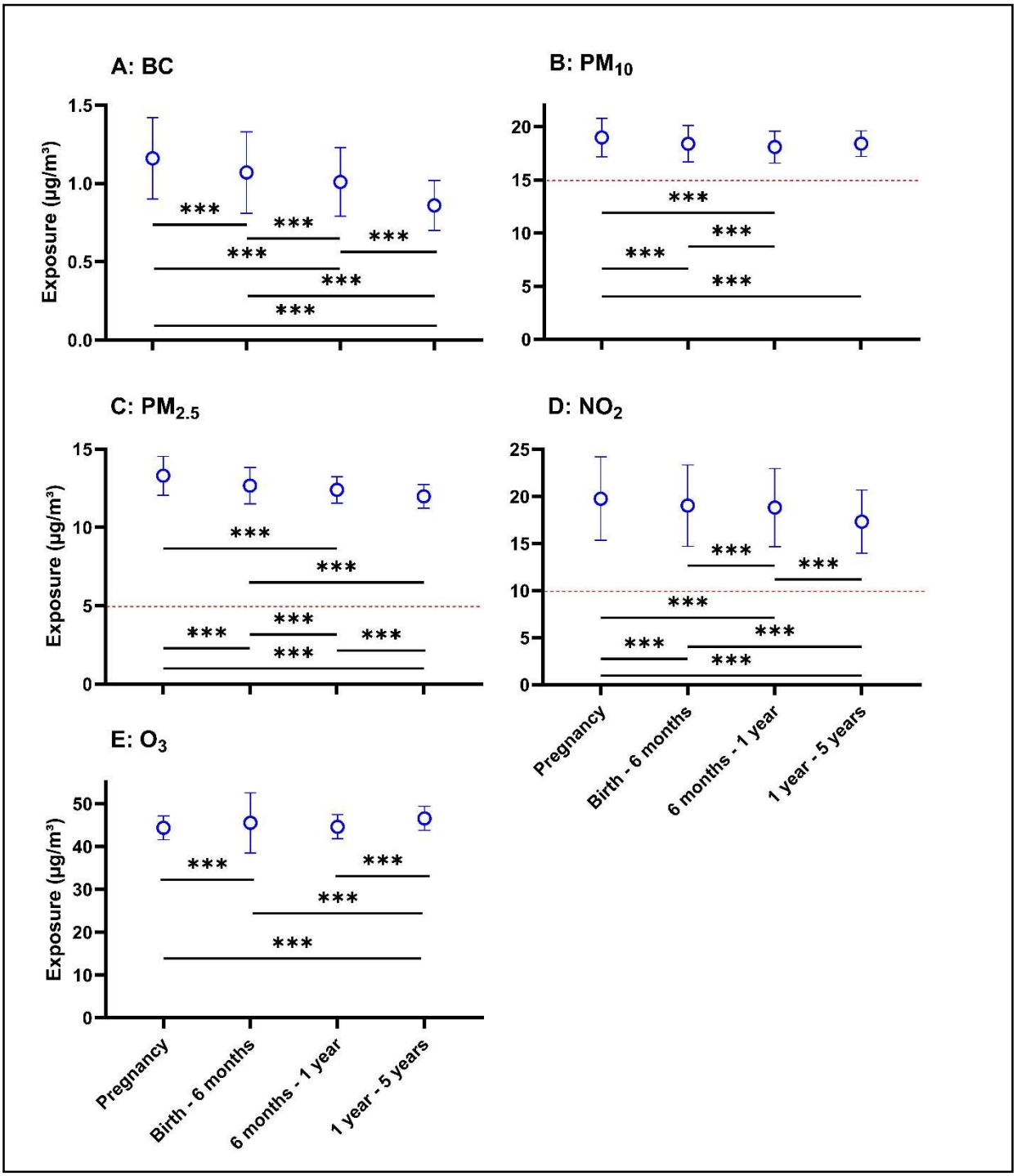


Figure 2: Air pollution exposure during the different periods: pregnancy, birth to six months old, six months old to one year old, one year old to five years old. Exposures are depicted with standard deviation as error bar. Recommendation of WHO for maximal annual exposure is shown as a dashed line. No recommendation for annual mean of exposure present for black carbon (BC) and O_3. Singificant differences are depicted with uninterupted lines with p-values indicated above. p-values generated using paired t-tests between the air pollution factors over the different periods: between pregnancy, six months old, one year old, and five years old. **A.** for BC. **B.** for PM_{10}, **C.** for $PM_{2.5}$. **D.** for NO_2. **E.** for O_3. PM_{10}, particulate matter with aerodynamic diameter ≤ 10 µm; $PM_{2.5}$, particulate matter with aerodynamic diameter ≤ 2.5 µm; WHO, World Health Organisation; *, p<0.05; **, p<0.01; ***, p<0.001.

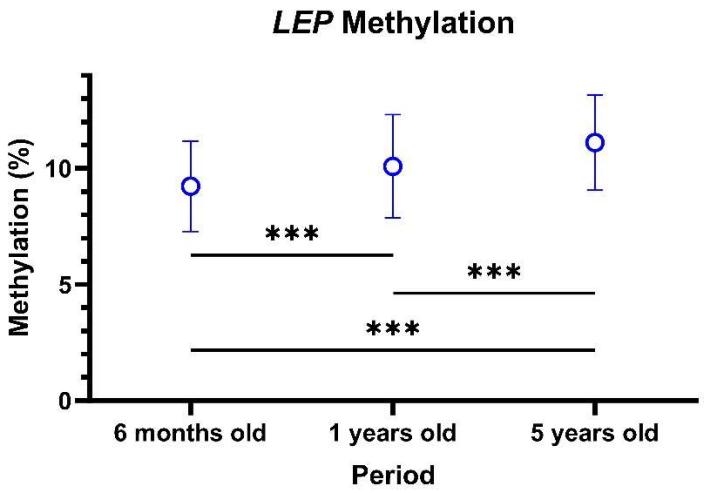
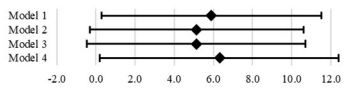
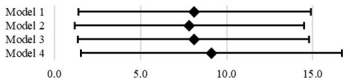
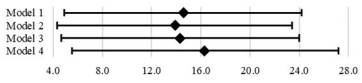
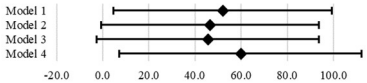
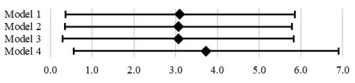
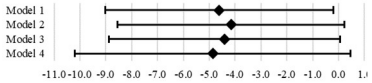
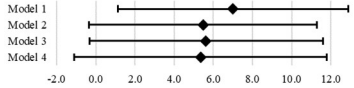
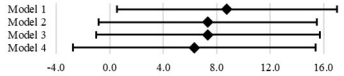
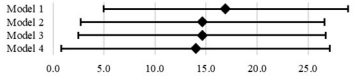
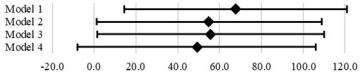
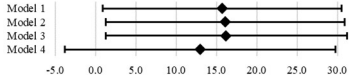
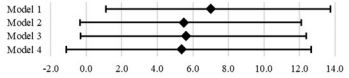
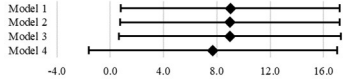
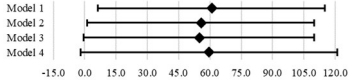


Figure 3. Mean *LEP* DNA methylation levels at six months old, one year old and five years old in study population. Mean *LEP* methylation levels for the different time points of this study: six months, one year, and five years of age, are presented as average *LEP* methylation in percentage with error bars indicating the standard deviations. p-values for differences over time derive from paired t-tests or paired Wilcoxon signed rank tests. *LEP*, leptin; ***, p<0.001.

TABLES

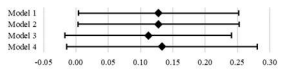
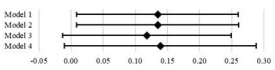
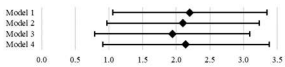
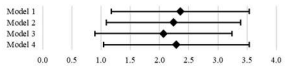
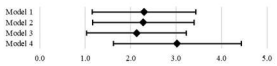
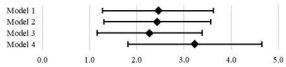
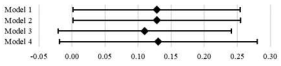
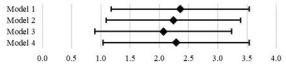
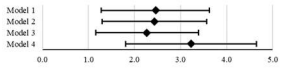
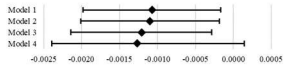
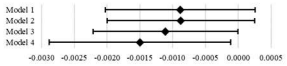
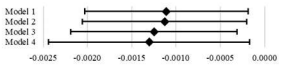
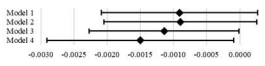
Table 1. Estimated effect sizes of significant associations of air pollution on growth factors.

	PM_{10}	$PM_{2.5}$	BC	NO_2	O_3
	Pregnancy				
Impedance longitudinal		/	/	/	/
Impedance 6m	NS	/	/	/	/
Impedance 1y					
	Birth-6m				
Impedance longitudinal		/	/	/	/
Impedance 6m				NS	NS
Impedance 1y	NS		NS	NS	NS
	6m-1y				
Impedance longitudinal		/	/	/	/
Impedance 1y			NS	NS	NS

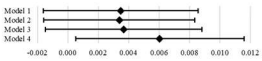
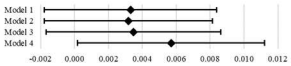
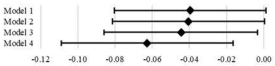
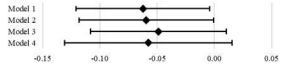
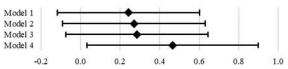
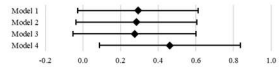
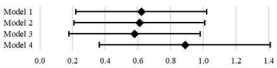
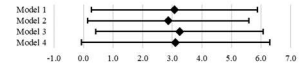
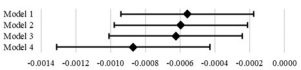
Significant estimated effects of air pollution on growth and mean *LEP* methylation during the indicated periods are presented. Data is shown as estimate (◆) (95% confidence interval (CI) (⊣⊢)). Estimated effect sizes and CI determined using linear mixed effects models or linear models. **A.** for impedance. **B.** for waist-hip-ratio and waist circumference. No other growth parameters showed significant associations. *LEP*: leptin; PM: particulate matter; BC: black carbon; 6m: six months old; 1y: one year old; 5y: five years old, NS: not significant.

Table 2. Estimated effect sizes of significant associations for environmental parameters on growth factors.

A

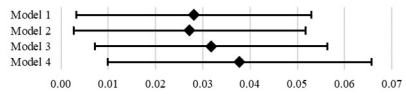
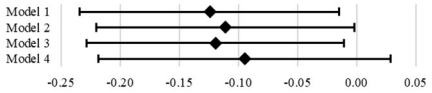
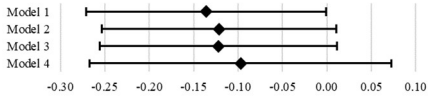
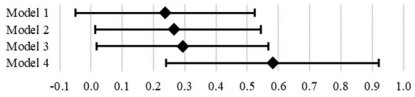
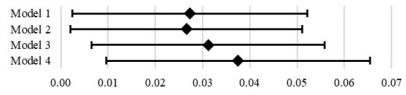
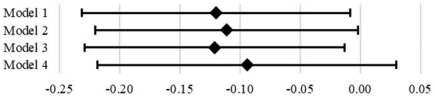
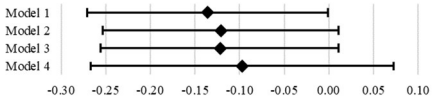
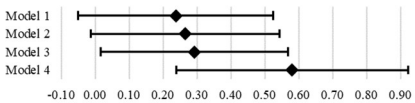
	WFL longitudinal	WFL 5y	BMI longitudinal	BMI 5y
Greenness factor PC2 6m		/		/
Urban green 6m	/		/	
Urban forest 6m	/		/	
Greenness factor PC2 1y	NS	/		/
Urban green 1y	/	/	/	
Urban forest 1y	/	/	/	
Forest distance 5y				

B

	Impedance longitudinal	Waist-hip-ratio longitudinal/5y	Fat percentage longitudinal/5y
Greenness factor PC2 6m	NS		NS
Greenness factor PC2 1y	NS		NS
Greenness factor PC1 5y	NS	NS	
Greenness factor PC2 5y	NS	NS	
Green in district 5y	/	/	
Urban green 5y	/	/	
Urban forest 5y	/	/	
Walkability 5y		NS	NS
Forest distance 5y	NS	NS	

Significant estimated effects of air pollution on growth during the indicated periods are presented. Data is shown as estimate (β) (95% confidence interval (CI)). Estimated effect sizes and CI determined using linear mixed effects models or linear models. **A.** For WFL and BMI. **B.** for other growth parameters. WFL: weight for length; BMI: body mass index; PC: principle component; 6m: six months old; 1y: one year old; 5y: five years old; NS: not significant.

Table 3. Estimated effect sizes of significant associations for environmental parameters on *LEP* methylation.

	Longitudinal	**1y**
Greenness factors PC2 6m		NS
Green in district 6m	/	
Small urban green 6m	/	
Urban forest 6m	/	
Greenness factors PC2 1y		NS
Green in district 1y	/	
Small urban green 1y	/	
Urban forest 1y	/	

Significant estimated effects of air pollution on mean leptin (*LEP)* methylation during the indicated periods are presented. Data is shown as estimate (β) (95% confidence interval (CI)). Estimated effect sizes and CI determined using linear mixed effects models or linear models. *LEP*: leptin; PC: principle component; 6m: six months old; 1y: one year old; 5y: five years old; NS: not significant.

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DECLARATION OF CONFLICTING INTERESTS

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.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Evi De Ryck: Conceptualisation, data curation, formal analysis, investigation, methodology, software, validation, visualisation, writing – Original draft, review, editing. **Manosij Ghosh:** Supervision, writing – review, editing. **Tim Nawrot:** Resources, writing – review, editing. **Brigitte Reimann:** Resources. **Gudrun Koppen:** Writing – review. **Els Verachtert:** Resources, writing – review, editing. **Roland Devlieger:** Writing – review, editing. **Lode Godderis:** Writing – review, editing. **Sara Pauwels:** Conceptualisation, data curation, funding acquisition, project administration, resources, supervision, writing – review, editing.

ADDITIONAL INFORMATION

Figures and tables:

Figure 1: 1.5-column fitting image

Figure 2: 2-column fitting image

Figure 3: 1.5 column fitting image

Table 1: 2-column fitting image

Table 2: 2-column fitting image

Table 3: 2-column fitting image

No color in print needed

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