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Green space exposure and blood DNA methylation at birth and in childhood – A multi-cohort study

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ABSTRACT

Green space exposure has been associated with improved mental, physical and general health. However, the underlying biological mechanisms remain largely unknown. The aim of this study was to investigate the association between green space exposure and cord and child blood DNA methylation.

Data from eight European birth cohorts with a total of 2,988 newborns and 1,849 children were used. Two indicators of residential green space exposure were assessed: (i) surrounding greenness (satellite-based Normalized Difference Vegetation Index (NDVI) in buffers of 100 m and 300 m) and (ii) proximity to green space

Abbreviations: 450K, Illumina Infinium HumanMethylation450 BeadChip; ADAMTS2, ADAM Metallopeptidase With Thrombospondin Type 1 Motif 2; BMI, Body mass index; CNP, 2′,3′-Cyclic Nucleotide 3′- Phosphodiesterase; CpG, Cytosine-phosphate-guanine; DataSHIELD, Data Aggregation Through Anonymous Summary-statistics from Harmonised Individual-level Databases; DMP, Differentially methylated position; DMR, Differentially methylated region; DNA, Deoxyribonucleic acid; DNaseI, Deoxyribonuclease I; ELAVL2, ELAV Like RNA Binding Protein 2; EPIC, Illumina Infinium MethylationEPIC BeadChip; eQTM, Expression Quantitative Trait Methylation; EWAS, Epigenome-wide association study; FDR, False-discovery rate; GWAS, Genome-wide association study; GO, Gene Ontology; KCNQ1DN, KCNQ1 Downstream Neighbor; KEGG, Kyoto Encyclopedia of Genes and Genomes; NDVI, Normalized Difference Vegetation Index; $PM_{2.5}$, Particulate matter with aerodynamic diameter <2.5 μm; PTPRN2, Protein Tyrosine Phosphatase Receptor Type N2; RNA, Ribonucleic acid; SDK1, Sidderick Cell Adhesion molecule; SLC6A12, solute carrier family 6-member 12.

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(having a green space ≥ 5,000 m^2 within a distance of 300 m). For these indicators we assessed two exposure windows: (i) pregnancy, and (ii) the period from pregnancy to child blood DNA methylation assessment, named as cumulative exposure. DNA methylation was measured with the Illumina 450K or EPIC arrays. To identify differentially methylated positions (DMPs) we fitted robust linear regression models between pregnancy green space exposure and cord blood DNA methylation and between cumulative green space exposure and child blood DNA methylation. Two sensitivity analyses were conducted: (i) without adjusting for cellular composition, and (ii) adjusting for air pollution. Cohort results were combined through fixed-effect inverse variance weighted meta-analyses. Differentially methylated regions (DMRs) were identified from meta-analysed results using the Enmix-combp and DMRcate methods.

There was no statistical evidence of pregnancy or cumulative exposures associating with any DMP (False Discovery Rate, FDR, p-value < 0.05). However, surrounding greenness exposure was inversely associated with four DMRs (three in cord blood and one in child blood) annotated to *ADAMTS2, KCNQ1DN, SLC6A12* and *SDK1* genes. Results did not change substantially in the sensitivity analyses.

Overall, we found little evidence of the association between green space exposure and blood DNA methylation. Although we identified associations between surrounding greenness exposure with four DMRs, these findings require replication.

1. Introduction

The notion that natural environments provide advantages for human beings is not novel, and, indeed an increasing body of evidence has established health benefits of these vegetation rich environments, commonly referred to as green space (Zare Sakhvidi et al., 2023). Access to green spaces has decreased due to the increasing urbanization, with more than half of the population, including 1.5 billion children, now living in urban areas (Suchitra, 2021; United Nations, 2018). In European cities, it has been estimated that a large number of premature deaths could be prevented by increasing exposure to green space (Barboza et al., 2021; Iungman et al., 2023).

Previous studies found associations of exposure to higher levels of residential green space with lower risk of pregnancy complications (Liao et al., 2019; Zanini et al., 2020), improved birth outcomes (Hu et al., 2021; Torres Toda et al., 2022), and enhanced physical and mental health during childhood, including better school performance and reduced risk of ADHD, stress, anxiety and depression (Zare Sakhvidi et al., 2022, 2023). Moreover, a nature-based intervention where participants were engaged with wetland nature confirmed some of the previous results (Maund et al., 2019).

Several potential pathways have been proposed to link green space to health. Green space can reduce stress, promote social contacts, increase physical activity (lower body mass index), mitigate exposure to air pollution, noise, and heat, or enrich the microbiome, among others (Bowyer et al., 2022; Markevych et al., 2017; Rook, 2013). Green space exposure, either directly or through these pathways, may affect the epigenome, which is defined as the sum of all modifications to DNA, or to DNA-associated RNA and proteins, that permit interpretation of the genome to instruct cell identity and function, and thus ultimately gene expression (Hemberger et al., 2020). Among all epigenetic marks, DNA methylation, the addition of a methyl group to the C5 position of the cytosine within a cytosine-guanine (CpG) dinucleotide, has been the most widely investigated in epidemiological settings.

In recent years, a few epigenome-wide association studies (EWASs) have assessed the relationship between exposure to green space and DNA methylation in adult blood (Jeong et al., 2022; Xu et al., 2021b), cord blood (Alfano et al., 2023) and child blood (Lee et al., 2021). However, all these studies were limited in sample size.

Here, we aimed to investigate the association between pregnancy green space exposure and cord blood DNA methylation and between green space exposure from pregnancy to child blood DNA methylation (abbreviated as cumulative exposure from now on) and child blood DNA methylation in a multi-cohort study.

2. Methods

2.1. Study population

A total of eight independent European birth and/or child cohorts from nine countries that had data on indicators of green space exposure and DNA methylation were included in this study: Avon Longitudinal Study of Parents and Children (ALSPAC), United Kingdom (Boyd et al., 2013; Fraser et al., 2013), Etude des Déterminants du développement et de la santé de l'Enfant (EDEN), France (Heude et al., 2016), the ENVIRonmental influence ON early AGEing (ENVIRONAGE; 2 subcohorts), Belgium (Janssen et al., 2017), the Generation R Study (Generation R), the Netherlands (Kooijman et al., 2016), Human Early-Life Exposome project (HELIX; including six jointly analyzed subcohorts), France, Greece, Lithuania, Norway, Spain and United Kingdom (Maitre et al., 2018), Infancia y Medio Ambiente (INMA), Spain (Guxens et al., 2012) and Piccolipiù cohort (Piccolipiù), Italy (Farchi et al., 2014). Full details of these studies are provided in Appendix A: Table S2A-C and Appendix B: Supplementary methods.

For the association of pregnancy green space exposure and cord blood DNA methylation, we had data on 2,988 mother-infant pairs from seven cohorts (ALSPAC, EDEN, ENVIRONAGE 450K, ENVIRONAGE EPIC, Generation R, INMA and Piccolipiù). For the association of cumulative green space exposure and child blood DNA methylation, we had data on 1,849 children from three studies (ALSPAC, Generation R and HELIX). We restricted the analyses to participants of European ancestry as the percentage of non-European individuals with DNA methylation data was less than a 10 % in each of the cohorts. Analyses were limited to singleton children and for non-twin siblings, only one child per mother was included. All cohorts acquired ethics approval through local ethics committees and informed consent was obtained for all participants prior to data collection (Appendix B: Supplementary methods).

2.2. Indicators of exposure to green space

The assessment of exposure to green space was conducted following a standardised protocol across all cohorts as part of the Lifecycle (Jaddoe et al., 2020) and ATHLETE (https://athleteproject.eu/) projects. Detailed methods for each cohort are provided in Appendix A: TableS2C and Appendix B: Supplementary methods. Briefly, to assess exposure to green space, we characterized two aspects of such exposure: (i) residential surrounding greenness and (ii) residential proximity to green space.

To characterize residential surrounding greenness, we applied the satellite-based Normalized Difference Vegetation Index (NDVI), which quantifies vegetation by measuring the difference between near-infrared (which vegetation strongly reflects) and red light (which vegetation

absorbs). NDVI values vary from −1 to 1, with higher values indicating more photosynthetic capacity (Tucker, 1979). This index was estimated using LANDSAT data at 30 m x 30 m resolution data captured during the greenest period of the year (summer in Northern cohorts and spring in Southern cohorts). Negative values in the images, which correspond to water and other non-green land cover, were all reclassified to null values. We assessed residential surrounding greenness as the average of NDVI across buffers of 100 m (NDVI 100 m) and 300 m (NDVI 300 m) around the residential addresses of the mother. These buffers were chosen i) based in previous evidence of the association between greenness within 100 and 300 m buffers and birth outcomes (Nieuwenhuijsen et al., 2019; Torres Toda et al., 2022) and ii) to capture data from the immediate surroundings of the residence (about 5 or 10 min around the house) (WHO Regional Office for Europe, 2016), which is the space where individuals are expected to spend the most time. Residential surrounding greenness within 500 m buffer was available but excluded as it was highly correlated with the 300 m buffer. NDVI indicators were estimated for two time periods: for pregnancy and for the period that goes from pregnancy to the age of child blood DNA methylation assessment (cumulative exposure). To quantify exposure during pregnancy, we used the measurement calculated at the residential address during the mother's pregnancy or at the time of birth (i.e. within studies this could be measured at any gestational age or at the date of delivery). If the mother moved house during pregnancy, we used the average weighted by time spent on each address. To estimate the cumulative exposure, we first calculated the average greenness at the residential addresses in up to four time-periods from pregnancy until age of child DNA methylation assessment: pregnancy, infancy (>0 to <= 2 years), early childhood (>2 to <= 6 years), and late childhood (>6 to <=10 years). Then, the means for each time period were averaged to get the cumulative exposure (Appendix A: Table S1; Appendix B: Supplementary methods). A minimum of two time-periods were required (pregnancy and at least one postnatal time-period). ALSPAC and Generation R had repeated yearly measurements of green space up to the assessment of DNA methylation. The HELIX study, which is composed of six European birth cohorts, had different number of measurements from pregnancy to childhood depending on the cohort. See Appendix A: Table S1; Appendix B: Supplementary methods for more details on how the average was calculated in each of the subcohorts.

In addition, for the pregnancy period, we calculated another indicator, the residential proximity to green space, which was defined as having a green space of equal to or larger than 5,000 m^2 within a distance of 300 m from the residential address (yes/no) (The WHO Regional Office for Europe, 2017). This indicator was calculated using the Europe-wide Urban Atlas (European Environment Agency, 2010). Cohorts with less than 10 individuals in one of the categories were excluded from this analysis, resulting in 2,318 participants from four cohorts (ALSPAC, Generation R, INMA, and Piccolipiù) (Fig. 1). Proximity to green space was not assessed for the period from pregnancy to childhood as we did not find a convincing way to estimate a categorical variable from repeat measures.

2.3. DNA methylation measurements

DNA was extracted from cord blood or child blood as indicated in the Appendix B: Supplementary methods. DNA methylation was measured using the Illumina Infinium 450K or EPIC array in cord blood and/or child blood. Each cohort conducted their own sample processing, quality control and normalization of DNA methylation data, as detailed in Appendix B: Supplementary methods. To reduce the impact of severe outliers in the DNA methylation data, cohorts winsorized the methylation beta values for 1% of the participants per CpG, 0.5% at the upper and lower ends of the distribution (Ghosh & Vogt, 2012). Methylation data were expressed as beta values, ranging from 0 (fully unmethylated) to 1 (fully methylated).

2.4. Covariates

The following variables were considered potential confounders between green space exposure and blood DNA methylation and added as covariates in the models: family socioeconomic position (assessed through maternal education), residential area deprivation, and maternal age (years). The maternal level of education (high, medium, low) was based on the highest ongoing or completed education at the time of delivery education. This categorization followed the International Standard Classification of Education 97/2011 (ISCED-97/2011) (UNESCO Institute for Statistics, 2012). Country specific indices of deprivation were used to create the area-level deprivation index (a multidimensional evaluation of an area's socioeconomic average conditions adjusted in thirds; low deprived; medium deprived, high deprived) (detailed information in Appendix A: Table S2C). Child's sex and age, the later only in the child blood DNA methylation analyses, were also added as covariates in the models to gain precision.

Due to the strong relationship between tobacco smoke and DNA methylation (Joubert et al.,2016), and in addition to the potential role of tobacco as a proxy of socio-economic confounders, we decided to adjust the models for this variable. Maternal smoking during pregnancy was self-reported and categorized in two levels: any maternal smoking during pregnancy (No/Yes).

Blood cellular composition might mediate the effect of green space exposure on DNA methylation, because of this, models adjusted and unadjusted for cellular composition were run. Cord blood cellular composition was estimated using the Gervin and Salas reference panel (Gervin et al., 2019), the IDOL algorithm for selection of CpGs (Koestler et al., 2016), and the constrained projection-quadratic programming algorithm by Houseman for deconvolution of 7 main blood cell types (CD8T, CD4T, NK, Bcell, Mono, Gran, nRBC) (Houseman et al., 2012). Child blood cellular composition was calculated using the Reinius reference panel (Reinius et al., 2012) with the pickCompProbes method for CpG selection (minfi R package), and the Houseman algorithm for deconvolution of 6 main blood cell types (CD4T, CD8T, NK, Bcell, Mono, Gran) (Houseman et al., 2012).

Given the ongoing controversy whether air pollution is a mediator or a confounder of green space exposure (Markevych et al., 2017), we run adjusted and unadjusted models for air pollution. The average exposure to particulate matter with an aerodynamic diameter < 2.5 μg/m3

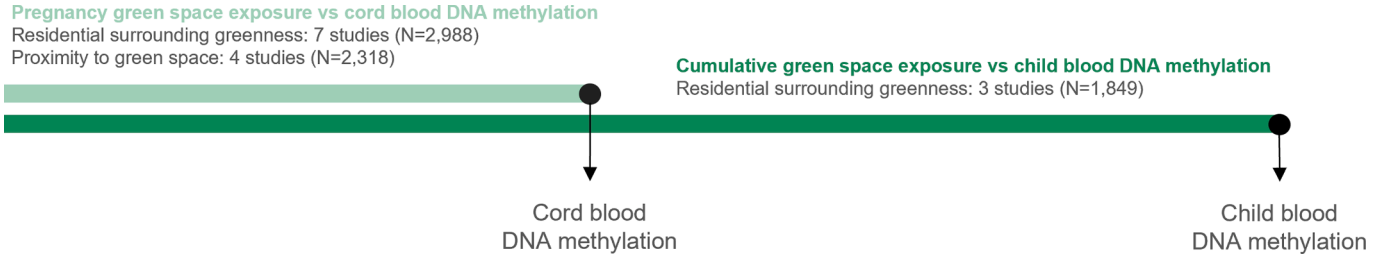

Fig. 1. Analyses scheme.

($PM_{2.5}$) was used as proxy of air pollution.

Finally, cohorts had the option to adjust the models for technical batch variables or ancestry within Europeans. In particular, Generation R and Piccolipiù adjusted the models for batch and HELIX for ancestry estimated as the first ten GWAS principal components (see Appendix B: Supplementary methods for more details).

2.5. Cohort-specific epigenome-wide association study (EWAS)

Six cohorts conducted the EWAS analyses locally following the same, prespecified statistical code (ALSPAC, ENVIRONAGE 450K, ENVIRONAGE EPIC, Generation R, HELIX and Piccolipiù) while the leading teams analysed two of the cohorts (EDEN, INMA) through Data Aggregation Through Anonymous Summary-statistics from Harmonised Individual-level Databases (DataSHIELD) (Gaye et al., 2014), an innovative federated platform that allows non-disclosive analysis of individual-level data. The INMA cohort was also analysed locally to validate the results obtained through DataSHIELD, showing consistent findings. Residential surrounding greenness (NDVI 100 m and NDVI 300 m) was standardized by dividing it by its interquartile range (IQR) in order to report the change in DNA methylation per IQR change in greenness. Robust linear regression models were fitted to evaluate the association between exposure to green space and DNA methylation using the limma R package (Ritchie et al., 2015) or the dsOmics R package (https://github.com/isglobal-brge/dsOmicsClient) in the case of DataSHIELD.

For the cord blood EWAS, the main models were adjusted for child sex, maternal education, neighbourhood SES, maternal age, smoking during pregnancy and cord blood cellular composition. For the child blood EWAS, models were additionally adjusted for child age and child blood cellular composition instead of cord blood cell composition (see section on covariates for details). See Appendix A: Table S3 for an overview of all models performed.

Two sensitivity analyses were conducted. First, the regression models were additionally adjusted for $PM_{2.5}$ during pregnancy (for cord blood) or cumulative pregnancy and childhood period (for child blood) (detailed information in Appendix B: Supplementary methods). Second, main models were run without adjusting for cellular composition to investigate its effect in the association.

Finally, we ran additional analyses adjusting for local climate (temperature and relative humidity transformed to non-linear terms) in two of the cohorts (INMA and Generation R). For the pregnancy analyses, we used the average temperature and relative humidity during pregnancy; For the childhood analyses, we calculated cumulative temperature and relative humidity variables as we did for the exposure to greenness (Appendix B: Supplementary methods).

2.6. Quality control and EWAS meta-analysis

We performed the quality control of the cohort-specific results for each model using the EASIER R package (ISGlobal-BRGE/EASIER: Tools for Methylation Data Analysis, 2022) (Appendix A: Table S4A-B). This included examining inflation and the distribution of effect estimates, standard errors and p-values and creating precision plots by plotting 1 divided by the median of the effect SE against the square root of the sample size for each cohort. We excluded control probes, non-CpG probes, probes that mapped to X/Y chromosomes, probes with poor base pairing quality (lower than 40 on 0–60 scale), probes with non-unique 30 bp 3′-subsequence (with cross-hybridizing problems), Infinium II probes with SNPs of global MAF over 1% affecting the extension base, probes with a SNP in the extension base that causes a color channel switch from the official annotation (Zhou et al., 2017) and probes that have shown to be unreliable in a recent comparison of the Illumina 450K and EPIC BeadChips (Fernandez-Jimenez et al., 2019). The percentage of probes removed in each cohort ranged between 3.9–11.9% (Appendix A: Table S4A-B).

To identify differentially methylated positions (DMPs), cohort-specific EWAS results were combined through fixed-effects inverse variance-weight meta-analyses (EASIER R package) at ISGlobal. Shadow meta-analyses using the Metal program (Willer et al., 2010) were conducted independently at the Erasmus Medical Center Rotterdam and results were compared. ENVIRONAGE EPIC and Piccolipiù were the only cohorts that used the Illumina Infinium EPIC array, thus only the EPIC array CpG sites overlapping the CpG sites in the 450K array (used in all the other cohorts) were meta-analysed. Furthermore, we included only those CpG sites present in at least 50% of the cohorts. The final number of CpG sites included in each meta-analysis is provided in Appendix A: Table S3.

Results were corrected for multiple testing using the false discovery rate (FDR) method (Benjamini, 2010). Genome-wide significance was defined at FDR p-value < 0.05 and suggestive significance at nominal p-value $< 1 \times 10^{-5}$. Effect sizes represent the percentage DNA methylation difference per interquartile range increase in residential surrounding greenness indicators (NDVI 100 m, NDVI 300 m) and the percentage DNA methylation difference for having residential proximity to a green space (green proximity). We calculated the I^2 statistic to explore heterogeneity across cohorts (Higgins & Thompson, 2002). Quality control of the meta-analysed results was also performed by calculating lambda and QQ-plots of p-values and volcano plots. Finally, leave-one-out analysis, in which we re-ran the main analysis repeatedly with one of the cohorts removed each time, was conducted to explore if any of the studies was unduly influencing the findings.

2.7. Differentially methylated regions (DMR)

Differentially methylated regions (DMRs) were explored using DMRcate (Peters et al., 2015) and Enmix-combp (Niu & Taylor, 2023), both R packages, on the meta-analysed results. DMRcate identifies DMRs from a tunable kernel smoothing process of association signals, and Enmix-combp identifies DMRs by combining low p-values of CpGs in an adjacent region of CpGs. Both packages use regression coefficients and standard deviations as input, in addition to uncorrected p-values for DMRcate (lambda = 1000; C = 2) and uncorrected p-values and chromosomal locations of each CpG for Enmix-combp (bin size = 310; seed = 0.05). We considered DMRs to be those detected after multiple-testing correction with both methods (Siddak p-value for Enmix-combp and FDR p-value for DMRcate < 0.05), with a minimum of one CpG in common and three consecutive CpGs within the DMR. DMRs were annotated using matchGenes in the Bumphunter R package (Jaffe et al., 2012). Finally, DMRs were explored on the leave-one-out meta-analysed results to examine if any of the studies was influencing the findings.

2.8. Follow-up analyses

To assess whether methylation levels of DMPs and DMRs were associated with the expression levels of nearby genes in child blood, we consulted the HELIX Expression Quantitative Trait Methylation (eQTM) catalogue (Arenas et al., 2022) (https://helixomics.isglobal.org/). Moreover, we checked whether the suggestive DMPs and CpGs within the DMRs had previously been associated with exposures or health traits using the EWAS catalogue (Battram et al., 2022b) and the EWAS Atlas (Li et al., 2019) databases. We also compared the list of suggestive DMPs and DMRs with previously reported studies evaluating the association between exposure to green space and blood DNA methylation (Alfano et al., 2023; Jeong et al., 2022; Lee et al., 2021; Xu et al., 2021b).

We conducted functional enrichment analyses of the suggestive DMPs (p-value $< 1x10^{-5}$) for Gene Ontology (GO) terms and pathways of the Kyoto Encyclopedia of Genes and Genomes (KEGG) using the missMethyl method (Phipson et al., 2016) as implemented in EASIER R package (ISGlobal-BRGE/EASIER: Tools for Methylation Data Analysis, 2022). Finally, we used eFORGE version 2.0 to examine enrichment for tissue-specific DNaseI hypersensitivity regions (Breeze et al., 2019).

3. Results

3.1. Study population

We meta-analysed results from seven independent cohorts (N = 2,988) with data on newborn DNA methylation status, and three studies with data on DNA methylation in children (N = 1,849). The description of the sociodemographic and lifestyle characteristics of the study participants and the levels of green space indicators are shown in Table 1; Appendix A: TableS2A-B.

The median age of the mothers ranged from 27 to 35 years, 18.5% to 46.3% lived in highly deprived areas and most of them did not smoke during pregnancy (>70%). In terms of cord blood analyses, the median gestational age in the cohorts ranged from 39.9 to 40.5 weeks. For the child blood analyses, the median age of the children in the cohorts ranged from 6.0 to 8.5 years.

For the pregnancy period, an increasing trend in the estimated residential surrounding greenness could be observed from the Southern to the Northern cohorts. In the Southern cohorts (INMA and Piccolipiù), the median NDVI values ranged from 0.2 to 0.3, while in the Northern countries the NDVI ranged from 0.4 to 0.6. The correlation coefficients between NDVI within a buffer of 100 m and a buffer of 300 m across cohorts ranged from 0.69 to 0.86 (Appendix C: Fig. S1). More than 70% of the participants had a green space within a distance of 300 m from their house. INMA had the lowest proximity to green space and Generation R had the highest. For the cumulative exposure, the median NDVI values varied from 0.4 in ALSPAC (United Kingdom) and Generation R (Netherlands) to 0.5 in HELIX, which includes children from France, Greece, Lithuania, Norway, Spain and the United Kingdom. The correlation coefficient of the cumulative exposure between the two buffers across cohorts ranged from 0.84 to 0.95. The correlation coefficient between NDVI during pregnancy and cumulative was very high for both buffers (between 0.88 and 0.95 for NDVI 100 m and between 0.88–0.96 for NDVI 300 m) (Appendix C: Fig. S2).

3.2. Green space during pregnancy and cord blood DNA methylation

3.2.1. DMP analyses

Lambda inflation factors for the main models of the association between pregnancy green space exposure and cord blood DNA methylation ranged from 0.97 to 1.10 (Appendix C: Fig. S3; Fig. S4; Fig. S5). After FDR correction, no DMPs were significantly associated with any of the green space indicators. At suggestive significance (p-value $< 1x10^{-5}$), eight, eight and three DMPs were associated with NDVI 100 m, NDVI 300 m and green proximity, respectively (Table 2; Fig. 2; Fig. S6). The full results can be found on the HELIX-omics Webpage (https://helixomics.isglobal.org/). Almost all (94.7%) of the suggestive hits showed low between-study heterogeneity ($I^2 < 0.5$) (Table 2). In the leave-one-out analyses, there was no strong evidence that any of the studies unduly influenced findings consistently across the suggestive DMPs (Appendix C: Fig. S7). Only one suggestive CpG (cg09223940) overlapped between NDVI 100 m and NDVI 300 m (Appendix C: Fig. S8). Pearson correlation coefficients of the effect estimates of genome-wide and suggestive CpGs across the three exposure variables are shown in Appendix C: Fig. S9. Coefficients of the association did not change substantially in the sensitivity analyses conducted for any of the three measures of green space. The median percentage change in the effect between the main and the sensitivity results was 5.0% for $PM_{2.5}$ adjustment and 8.0% for cellular composition adjustment (Appendix A: Table S5). Additional analyses in INMA and Generation R adjusting for temperature and relative humidity did not change the results substantially for any of the exposures of green space (Fig. S10).

3.2.2. DMR analyses

In cord blood, two and one DMRs were associated with pregnancy exposure to NDVI 100 m and NDVI 300 m, respectively. These DMRs were annotated to three unique genes (*ADAMTS2, KCNQ1DN* and *SL6A12*) and included from 3 to 43 CpGs with a width from 124 to 2,533 bp (Table 3). No DMRs were found for green proximity. Despite observing certain fluctuations in the results when excluding each of the studies in the leave-one-out analyses, we did not find strong evidence that any of the cohorts consistently had an impact on the significant DMRs (Appendix A: Table S6).

3.3. Cumulative green space exposure and child blood DNA methylation

3.3.1. DMP analyses

Cumulative residential surrounding greenness exposure was not associated with any DMP after FDR correction. At suggestive significance, one and five DMPs were identified, for NDVIs 100 m and 300 m, respectively (Table 2; Fig. 2; Fig. S6). The full results can be found in HELIX-omics webpage and in Zenodo, an EU-open research repository. Lambda inflation factors ranged from 0.94 to 0.96 (Appendix C: Fig. S11; Fig. S12). None of the suggestive DMPs overlapped between residential surrounding greenness in buffers of 100 m and 300 m (Appendix C: Fig. S13). Pearson correlation coefficients of the effect estimates of genome-wide and suggestive DMPs between the NDVI indicators were 0.83 and 0.96, respectively (Appendix C: Fig. S14). Fifty percent of the suggestive significant DMPs showed between-study heterogeneity ($I^2 > 0.5$) (Table 2). Regarding the leave-one-out analyses, none of the studies consistently unduly influenced findings across the suggestive DMPs, with less than a 20% change in effect each time that we excluded a study (Appendix C: Fig. S15). In the sensitivity analyses, the coefficients of the association did not change substantially. The median percentage change in the effect between the main and the sensitivity results was 8.2% for $PM_{2.5}$ adjustment and 6.7% for cellular composition unadjustment (Appendix A: Table S5). Additional results for residential surrounding greenness in Generation R did not change substantially after adjustments for temperature and relative humidity (Fig. S16). Ultimately, none of the suggestive DMPs for child blood were found among suggestive DMPs in cord blood, nor vice versa (Appendix C: Fig. S17).

3.3.2. DMR analyses

We identified one DMR associated with cumulative NDVI 300 m. This DMR was annotated to the *SDK1* gene, included a total of seven CpGs, and had a length of 1020 kb according to DMRcate method (Table 3).

3.4. Follow-up analyses

None of the suggestive DMPs or CpGs within the significant DMRs identified in child blood were described to eQTMs in child blood (Table S7). According to the EWAS Atlas and EWAS Catalogue, methylation levels at the suggestive DMPs or CpGs within the significant DMRs have previously been related to child age, sex, gestational age, preterm birth, autoimmune diseases, respiratory conditions, metabolic disorders, maternal BMI and environmental exposures such as air pollution or smoking (see Appendix A: Table S8 for detailed information).

Among the 26 unique significant DMPs reported in previous studies on the link between green space and DNA methylation, three (cg00809988, cg18311871 and cg04720477) were nominally significant (p-value < 0.05) and had the same direction in our study (Supplementary Table S9). Furthermore, out of the genes annotated to the DMRs found in this study, two (*ADAMTS2* and *KCNQ1DN*) had previously been reported (Alfano et al., 2023; Jeong et al., 2022) (Fig. 3). Whereas more than 70% of the CpGs in the DMR annotated to *KCNQ1DN* (chr11:2,889,602–2,891,495) overlapped with the CpGs in the DMR identified before (chr11:2,889,629–2,891,360 (Alfano et al., 2023) and chr11:2,889,886–2,891,495 (Jeong et al., 2022)), none of the CpGs within the DMR annotated to *ADAMTS2*

Table 1
Characteristics of the study populations included in the main meta-analyses.

			Green space indicators			Maternal characteristics and lifestyle		Sociodemographic		Child characteristics		Air pollution
			NDVI 100 m	**NDVI 300 m**	**Green proximity** (Yes)	**Maternal age** (Years)	**Smoking during pregnancy** (Yes)	**Maternal education** (Low)	**Neighborhood SES** (High deprivated area)	**Gestational age** (Weeks)/ **Child age** (Years)	**Sex** (Female)	**$PM_{2.5}$** (mg/m3)
Cohort	**Country**	**N**	**median (IQR)**	**median (IQR)**	**N (%)**	**median (IQR)**	**N (%)**	**N (%)**	**N (%)**	**median (IQR)**	**N (%)**	**median (IQR)**
Association of green space during pregnancy and cord blood DNA methylation (N = 2,988)												
ALSPAC	United Kingdom	618	0.4 (0.3–0.4)	0.4 (0.4–0.5)	466 (75.4 %)	27.0 (23.0–31.0)	88 (14.2 %)	38 (6.1 %)	177 (28.6 %)	40.5 (39.5–41.5)	317 (51.3 %)	13.2 (12.8–13.7)
EDEN[b]	France	137	0.5 (0.4–0.6)	0.6 (0.5–0.6)	–	29.0 (27.0–33.0)	35 (4.8 %)	7 (5.1 %)	36 (26.3 %)	40.4 (39.4–41.4)	59 (43.1 %)	16.2 (15.0–17.0)
ENVIRONAGE 450K	Belgium	188	0.5 (0.5–0.6)	0.6 (0.5–0.6)	–	29.0 (27.0–32.0)	25 (13.3 %)	26 (13.8 %)	65 (34.6 %)	40.0 (39.0–40.6)	90 (47.9 %)	12.9 (11.6–14.3)
ENVIRONAGE EPIC[a]	Belgium	345	0.5 (0.5–0.6)	0.6 (0.5–0.7)	–	30.0 (27.0–33.0)	39 (11.3 %)	25 (7.2 %)	87 (25.2 %)	39.9 (39.0–40.6)	177 (51.3 %)	14.8 (12.5–16.2)
Generation R	Netherlands	1171	0.4 (0.3–0.5)	0.4 (0.3–0.5)	981 (83.8 %)	32.0 (30.0–34.0)	276 (23.6 %)	23 (22.0 %)	542 (46.3 %)	40.3 (39.4–41.1)	581 (49.6 %)	20.5 (18.1–22.6)
INMA	Spain	357	0.2 (0.1–0.2)	0.2 (0.2–0.3)	252 (70.6 %)	32.0 (29.0–34.0)	108 (30.3 %)	98 (27.5 %)	66 (18.5 %)	39.9 (39.0–40.9)	176 (49.3 %)	15.1 (14.3–16.1)
Piccolipiù[a]	Italy	172	0.3 (0.2–0.4)	0.3 (0.3–0.4)	136 (79.1 %)	35.2 (31.6–37.8)	41 (23.8 %)	22 (12.8 %)	55 (32 %)	40.1 (39.0–41.0)[d]	84 (48.8 %)	12.2 (11.1–12.8)[d]
Association of cumulative green space and child blood DNA methylation (6–9 years) (N = 1,849)												
ALSPAC	United Kingdom	682	0.4 (0.4–0.5)	0.4 (0.4–0.5)	–	27.0 (23.0–31.0)	96 (14.1 %)	42 (6.2 %)	153(22.4 %)	7.4 (7.3–7.5)	349 (52.2 %)	12.8 (12.4–13.1)
Generation R	Netherlands	440	0.4 (0.3–0.5)	0.4 (0.4–0.5)	–	32.0 (30.0–34.2)	101 (23.0 %)	137 (31.1 %)[c]	182 (41.4 %)	6.0 (5.8–6.1)	230 (52.3 %)	18.4 (17.5–19.1)
HELIX	France, Greece, Lithuania, Norway, Spain, UK	727	0.4 (0.2–0.5)	0.5 (0.3–0.6)	–	31.3 (28.3–34.1)	127 (1.5 %)	387 (40.4 %)	263 (22.4 %)	8.5 (6.7–9.3)	326 (44.8 %)	15.0 (12.4–17.0)

All individuals were from European ancestry. We restricted the analyses to 450K array as only two cohorts were measured with EPIC array. Data are presented as median (Inter-quartile range) or count (%). IQR: inter-quartile range; NDVI 100 m: mean normalized difference vegetation index (NDVI) within a buffer of 100 m; NDVI 300 m: mean normalized difference vegetation index (NDVI) within a buffer of 300 m; Green proximity: have a green space of larger than 5,000 m^2 within a distance of 300 m; SES: socio-economic status; $PM_{2.5}$: Particulate matter with aerodynamic diameter < 2.5 μm.

[a] EPIC array.

[b] In the cord blood analyses, EDEN was excluded for the green proximity analysis due to < 10 individuals in one of the categories.

[c] Two categories of maternal education were combined (low and middle) as < 10 individuals were in the low maternal education level.

[d] Based on 105 out of 172 children included in the sensitivity analysis.

Table 2
Differentially methylated positions (DMPs) associated with green space indicators in cord and child blood (p-value < $1x10^{-05}$), ordered by P-value.

Association of green space during pregnancy and cord blood DNA methylation

Green space indicator	CpG	Chr	Position (GRCh37/ hg19)	N cohorts	N samples	Coefficient[a]	SE	P-value	FDR	Direction of the effect[b]	I^2	Gene	Location in gene	Relation to CpG islands
NDVI 100 m	cg26764250	8	104,090,700	7	2,988	0.4187	0.0856	1.02E-06	0.23	+++++++	0.00	–	–	OpenSea
	cg21554217	5	138,897,467	7	2,988	0.3147	0.0652	1.43E-06	0.23	+-++++	0.46	–	–	Island
	cg00455747	18	8,659,509	7	2,988	−0.2154	0.0452	1.91E-06	0.23	−−−−−−	0.15	–	–	Island
	cg12062099	1	85,527,597	7	2,988	0.352	0.0742	2.16E-06	0.23	+++++++	0.00	*WDR63*	TSS1500	OpenSea
	cg09223940[c]	14	65,095,871	7	2,988	−0.1392	0.0302	4.20E-06	0.36	−−−−−−	0.00	–	–	OpenSea
	cg22378919	1	119,522,188	6	1,817	−0.2315	0.0511	5.95E-06	0.42	−−?+-	0.70	*TBX15*	5′UTR	N_Shore
	cg06915343	2	240,029,626	7	2,988	−0.1865	0.0416	7.59E-06	0.42	−−−−−−	0.00	HDAC4	Body	N_Shore
	cg15002700	12	133,431,021	5	2,471	−0.1434	0.0321	7.92E-06	0.24	++-?–?	0.42	*CHFR*	Body	S_Shore
NDVI 300 m	cg00009927	16	1,157,223	6	2,816	−0.2981	0.0589	4.21E-07	0.18	−−−−−?	0.00	–	–	S_Shore
	cg25350136	19	44,617,363	7	2,988	−0.1431	0.0295	1.23E-06	0.25	−−−−−−	0.00	*ZNF225*	TSS200	N_Shore
	cg05406334	4	665,518	5	2,471	0.1935	0.0404	1.74E-06	0.25	+++?++?	0.00	–		N_Shore
	cg09223940[c]	14	65,095,871	7	2,988	−0.1628	0.0349	3.10E-06	0.30	+−−−−−−	0.00	–		OpenSea
	cg01800735	17	745,664	7	2,988	0.0913	0.0197	3.75E-06	0.30	+++++++	0.00	*NXN*	Body	S_Shore
	cg04674792	15	44,116,616	7	2,988	−0.2450	0.0533	4.40E-06	0.30	−−−−−−	0.00	*MFAP1*	Body	N_Shore
	cg21621910	2	102,486,284	7	2,988	−0.1112	0.0243	4.85E-06	0.30	+−−−−−−	0.43	*MAP4K4*	Body	OpenSea
	cg12658552	12	46,323,656	7	2,988	−0.2399	0.0537	7.95E-06	0.41	−−−−+-	0.00	*SFRS2IP*	Body	OpenSea
Green proximity	cg21465231	4	186,697,797	4	2,318	−0.3130	0.0659	2.06E-06	0.71	−−−	0.13	*SORBS2*	5′UTR; TSS1500	OpenSea
	cg26337816	11	132,582,640	4	2,318	−0.4389	0.0944	3.36E-06	0.71	−−−	0.00	*OPCML*	Body	OpenSea
	cg21037057	14	57,464,939	2	790	1.6289	0.3669	9.14E-06	0.91	+??+	0.28	–	–	OpenSea

Association of cumulative green space and child blood DNA methylation (6–9 years)

Green space indicator	CpG	Chr	Position (GRCh37/ hg19)	N cohorts	N samples	Coefficient[a]	SE	P-value	FDR	Direction of the effect[b]	I^2	Gene	Location in gene	Relation to CpG Islands
NDVI 100 m	cg03499581	15	78,384,868	3	1,849	−0.5060	0.0997	3.96E-07	0.17	−−–	0.00	*SH2D7*	TSS200	OpenSea
NDVI 300 m	cg22169990	7	150,786,051	3	1,849	−0.5444	0.1152	2.31E-06	0.53	−−–	0.73	*AGAP3*	Body	S_Shore
	cg09309085	20	40,706,193	3	1,849	−0.3921	0.0858	4.92E-06	0.53	−−–	0.76	*PTPRT*	3′UTR	OpenSea
	cg13976876	1	192,778,160	3	1,849	0.0430	0.0095	5.69E-06	0.53	+++	0.00	*RGS2*	TSS200	Island
	cg11909311	5	31,908,512	3	1,849	0.2122	0.0470	6.47E-06	0.53	+++	0.00	*PDZD2*	Body	OpenSea
	cg03050127	17	59,413,660	3	1,849	−0.4538	0.1020	8.79E-06	0.53	−-+	0.73	*BCAS3*	Body	OpenSea

SE: standard error; P-value: nominal p-value; FDR: False discovery rate; I^2: heterogeneity index across cohorts; CpG: cytosine-guanine dinucleotide; Chr: Chromosome; Position refers to Genome Research Consortium human genome build 37 (GRCh37)/UCSC human genome 19 (hg19);5′UTR: five prime untraslated region, refers to a part of promoter region on the right side of transcription starts site; 3′UTR: three prime untraslated region, refers to a part of promoter region on the left side of transcription starts site (gene transcription starts from left to right); TSS200: 0–200 bp upstream from transcription start site, refers to a part of promoter region; TSS1500: 200–1500 bp upstream from transcription start site, refers to a part of promoter region; Island: located in a CpG island; S_Shore: 0–2 kb downstream (3′) of a CpG island; N_Shore: 0–2 kb upstream (5′) of a CpG island; OpenSea: the rest of the genome.

[a] The regression coefficients represent % of DNA methylation difference per interquartile range increase in residential surrounding greenness indicators (NDVI 100 m, NDVI 300 m) and % of DNA methylation difference between categories of residential proximity to green space (Green proximity) in the main model.

[b] Order of the included cohorts in the meta-analysis: ALSPAC, EDEN, ENVIRONAGE 450K, ENVIRONAGE EPIC; Generation R, INMA, Piccolipiù (cord blood analysis). For Green proximity 3 cohorts were not included (EDEN, ENVIRONAGE 450 K, ENVIRONAGE EPIC) as they did not have green proximity; ALSPAC, Generation R, HELIX (child blood analysis).”?” Means that CpG was not measured in that cohort.

[c] Suggestive CpG sites associated with more than one green space indicator.

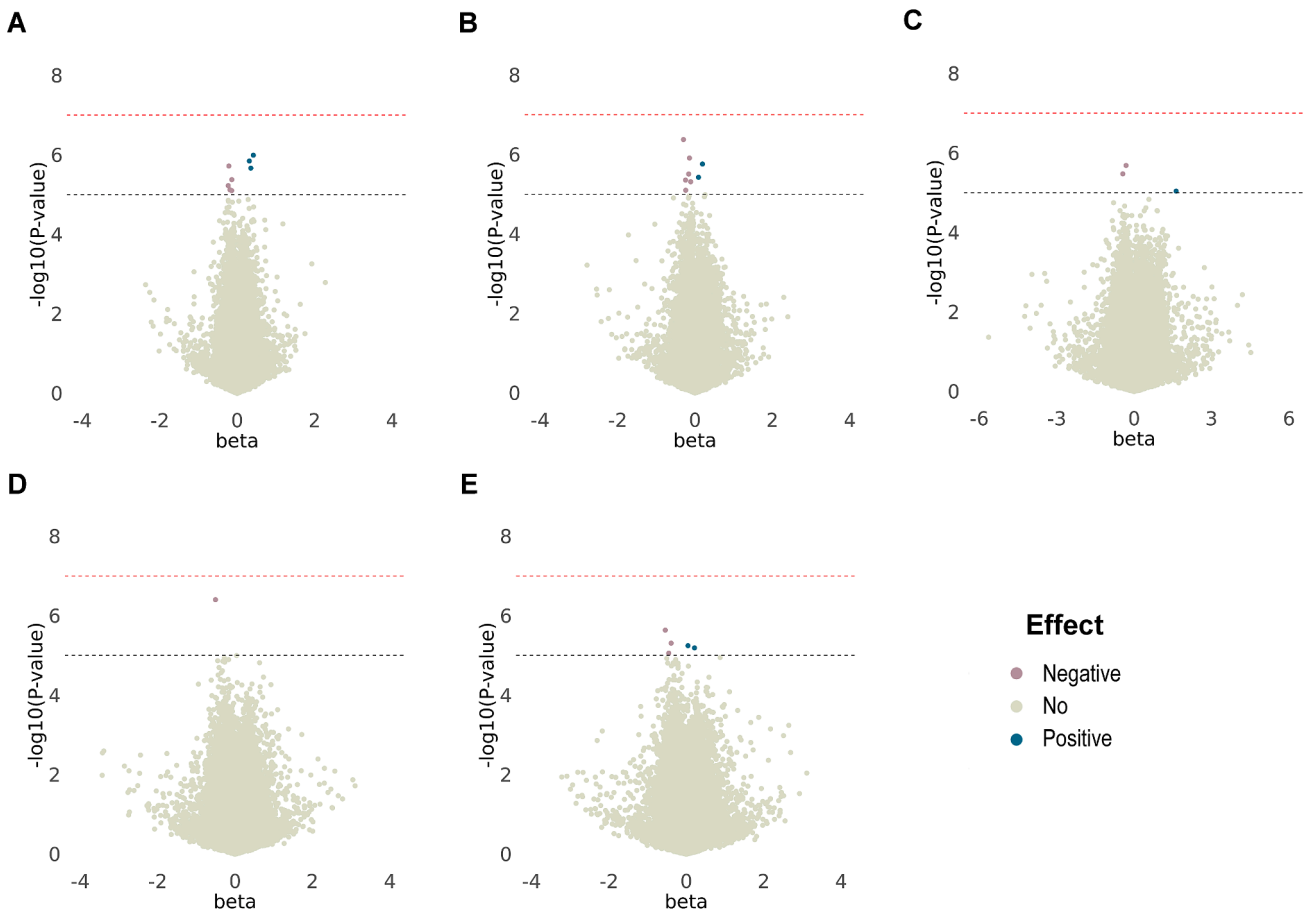

Fig. 2. Volcano plots showing the effect estimates (betas) on the x-axis and the ($-\log_{10}$) p-values on the y-axis for the associations between green space and blood DNA methylation at birth and during childhood. No association passed FDR corrected p-values < 0.05. The black line represents the suggestive p-value ($1x10^{-5}$) and the red line represents FDR (0.05) p-value threshold. (A) NDVI 100 m during pregnancy, (B) NDVI 300 m during pregnancy, (C) Green proximity during pregnancy, (D) Cumulative NDVI 100 m, (E) Cumulative NDVI 300 m. The effect estimates represent % of DNA methylation difference per interquartile range increase in residential surrounding greenness indicators (NDVI 100 m, NDVI 300 m) and % of DNA methylation difference between categories of residential proximity to green space (Green proximity).

(chr5:178,593,785–178,594,990) overlapped with the DMR identified in Alfano's study (chr5:178,547,863–178,548,373). The genomic context of these two DMRs is illustrated in Appendix C: Fig. S18.

Finally, no functional enrichment of suggestive DMPs was found for GO terms or KEGG pathways at FDR correction (Appendix A: Table S10-S13). Additionally, there was no enrichment for tissue-specific DNaseI hypersensitivity regions.

4. Discussion

In this multi-cohort study, we meta-analysed EWAS results from seven population-based cohorts across Europe to evaluate the association between pregnancy green space exposure and cord blood DNA methylation, as well as the EWAS results from three studies to assess the association between cumulative pregnancy and childhood green space exposure and child blood DNA methylation.

No genome-wide significant DMPs were found for any measures of pregnancy or cumulative exposure to green space. However, we identified associations between pregnancy residential surrounding greenness and three DMRs in cord blood annotated to *ADAMTS2, KCNQ1DN* and *SLC6A12*. Additionally, we found that cumulative residential surrounding greenness (300 m buffer) was associated with one DMR in child blood annotated to *SDK1*.

Four previous studies on green space and blood DNA methylation have been published to date. One evaluated exposure to greenness and blood methylation in 479 women from Australia and identified associations with one CpG and 35 DMRs (Xu et al., 2021b). Another included 982 women and men from the Switzerland and identified 219 DMRs (163 and 56 DMRs for NDVI within a buffer of 30 m and 500 m, respectively) (Jeong et al., 2022). Regarding children, Lee et al. analyzed the association of greenness with candidate CpGs previously associated with child intelligence quotient in 59 participants from a study from South Korea. From the analyzed CpGs, 25 were significantly associated with greenness exposure at age 2 years (Lee et al., 2021). Finally, only one study involving 538 newborns of the ENVIRONAGE cohort from Belgium, which is also part of this study, assessed associations between residential green space exposure during pregnancy and cord blood DNA methylation (Alfano et al., 2023), identifying one significant CpG and 147 DMRs.

Three of the significant DMPs (cg00809988, cg18311871, and cg04720477) found in the aforementioned studies (Lee et al., 2021; Xu et al., 2021b) were replicated with the same direction of the effect and nominal significance in our study. These CpGs, annotated *to ELAV Like RNA Binding Protein 2 (ELAVL2)*, *Protein Tyrosine Phosphatase Receptor Type N2 (PTPRN2)* and *2′,3′-Cyclic Nucleotide 3′- Phosphodiesterase (CNP),* showed an inverse association with greenness within 100 m or 300 m buffers. These genes have been observed to play a role in mental health (Al-Abdi et al., 2020; Curtis et al., 2011; Mulligan & Bicknell, 2023). In addition, the genes annotated to two of our DMRs, *ADAM Metallopeptidase With Thrombospondin Type 1 Motif 2 (ADAMTS2)* and *KCNQ1 Downstream Neighbor (KCNQ1DN),* have been identified in previous studies (Fig. 3) (Alfano et al., 2023; Jeong et al., 2022). Firstly, we

Table 3
Differentially methylated regions (DMRs) associated with green space indicators in cord and child blood (with at least three CpGs and identified with two methods), ordered by DMRcate FDR p-value.

		Enmix-combp				DMRcate						
Association of green space during pregnancy and cord blood DNA methylation												
Green space indicator	**Genomic location (GRCh37/hg19)**	**N CpGs**	**Siddak p-value**	**Width (bp)**	**Gene region**	**Genomic location (GRCh37/hg19)**	**N CpGs**	**Mean effect [a]**	**FDR p-value**	**Width (bp)**	**Gene region**	**Overlap (bp) [b]**	**Gene**
NDVI 100 *m*	chr11:2,890,389–2,890,726	22	1.2E-05	337	promoter	chr11:2,889,602–2,891,495	43	−0.002	2.0E-02	1893	overlaps 5′	337	*KCNQ1DN*
	chr5:178,594,526–178,594,650	3	5.3E-03	124	inside	chr5:178,593,785–178,594,990	10	−0.017	2.9E-02	1205	inside	124	*ADAMTS2*
NDVI 300 *m*	chr12:322,514–322,660	5	1.5E-04	146	inside	chr12:322,214–324,747	18	−0.037	4.2E-02	2533	overlaps 5′	146	*SLC6A12*
Association of cumulative green space and child blood DNA methylation (6–9 years)													
Green space indicator	**Genomic location (GRCh37/hg19)**	**N CpGs**	**Siddak p-value**	**Width**	**Gene region**	**Genomic location (GRCh37/hg19)**	**N CpGs**	**Mean effect[a]**	**FDR p-value**	**Width**	**Gene region**	**Overlap (bp)**	**Gene**
NDVI 300 *m*	chr7:4,218,743–4,218,991	3	1.5E-02	248	inside	chr7:4,218,154–4,219,174	7	−0.029	3.0E-02	1020	inside	248	*SDK1*

Only those DMRs detected after multiple-testing correction with both methods (Siddak p-value for Enmix-combp and FDR p-value for DMRcate < 0.05), with a minimum of one CpG in common and three consecutive CpGs within the DMR were considered. N CpGs: number of CpGs included in the DMR; FDR: False discovery rate; Width: width of the region (difference between the end and the start position); bp: base pair.

[a] Mean of coefficients of CpGs included in the region, where coefficients represent % DNA methylation difference per interquartile range increase in residential surrounding greenness indicators (NDVI 100 m, NDVI 300 m).

[b] Number of overlapped bp between the regions detected by both methods.

found that higher exposure to NDVI 100 m during pregnancy was associated with lower methylation at a DMR annotated to *ADAMTS2*. In the current study, this DMR contains ten CpGs while the DMR annotated to the same gene in Alfano's study comprises three CpGs, with none of them overlapping between the two studies (Alfano et al., 2023). In both cases, these DMRs are located in the *ADAMTS2* gene body, albeit in two different CpG islands. The three CpGs within the DMR from Alfano's paper showed a consistent effect direction in our meta-analysis results. While we assessed greenness using NDVI, Alfano's study examined a higher-resolution measure of greenness, enabling the classification of green space into three categories based on vegetation heigh: (1) high green space (>3m), (2) low green space (<3m), and (3) total green space, referring to overall vegetation cover. Although not entirely comparable, both studies found associations between greenness and DMRs annotated to *ADAMTS2*. *ADAMTS2* encodes an extracellular matrix protein that is mainly recognized for its role in cleaving the propeptides of collagen I and II (Colige et al., 2005) and also has an implication in the control of transforming growth factor (TGF)-beta activity (Bekhouche et al., 2016). This gene has been linked with connective tissue disorders (Van Damme et al., 2016) and brain diseases (Romay et al., 2019; Ruso-Julve et al., 2019). Furthermore, some of the CpGs within the DMR annotated to *ADAMTS2* have previously been related to autoimmune diseases according to the EWAS catalogue.

Secondly, we found an inverse association between NDVI 100 m and DNA methylation at a DMR located in *KCNQ1DN* gene. This region comprised a total of 43 CpGs, many of which were located upstream of the transcription start site (TSS), in the promoter region. The direction of effect was consistent with Alfano's study in cord blood (Alfano et al., 2023). However, in adults, the effect was in the opposite direction (Jeong et al., 2022). Out of the 43 CpG sites within the DMR identified in our study, 33 and 36 CpGs overlapped with the CpGs within the DMRs identified in cord blood and adult blood, respectively. *KCNQ1DN* is an imprinted gene expressed from the maternal allele (Xin et al., 2000) implicated in cell growth inhibition and cell cycle progression in renal cell carcinoma (Yang et al., 2019). Its DNA methylation levels, specifically at cg01530101 (included in the DMR), have been previously associated with aging (Koch & Wagner, 2011). Furthermore, most of the CpGs within the DMR annotated to *KCNQ1DN* have previously been related to age according to the EWAS catalogue. In order to verify that the overlap of these two genes with the findings from Alfano's study was not due to the presence of ENVIRONAGE in the cord blood analyses, we repeated the analyses excluding this cohort. In the results without ENVIRONAGE, the DMR annotated to *KCNQ1DN* was detected with the Enmix-combp method while the DMR annotated to *ADAMTS2* was not significant with neither of the two methods (Enmix-combp or DMRcate). However, results also fluctuated when excluding any of the cohorts of the study, showing that the DMR results are highly dependent on the cohorts analysed and need to be replicated.

The other two DMRs identified in our study had not previously been linked to green space exposure. The DMR annotated to the *Solute carrier family 6-member 12 (SLC6A12)* gene showed an inverse association with NDVI 300 m exposure during pregnancy. *SLC6A12* is a member of the neurotransmitter transporter family implicated in the cellular uptake of betaine and GABA in a sodium-and chloride (NaCl)-dependent process. This gene plays a role in kidney, brain and liver tissues, functioning as a methyl donor in the latter tissue (Kempson et al., 2014). This DMR was located in 5′ UTR of the gene. Finally, the DMR located in *Sidderick Cell Adhesion molecule 1* (*SDK1)* was inversely associated with cumulative exposure to NDVI 300 m. *SDK1* is implicated in the cell junction organization. CpGs within this region have been associated with age (Mulder et al., 2021).

According to literature, green space can be beneficial for health by reducing harm (decrease air pollution, noise and heat), restoring capacities (decrease stress and increase attention) and increasing physical activity and social contacts (Markevych et al., 2017). It has been hypothesised that NDVI within a buffer of 100 m may particularly

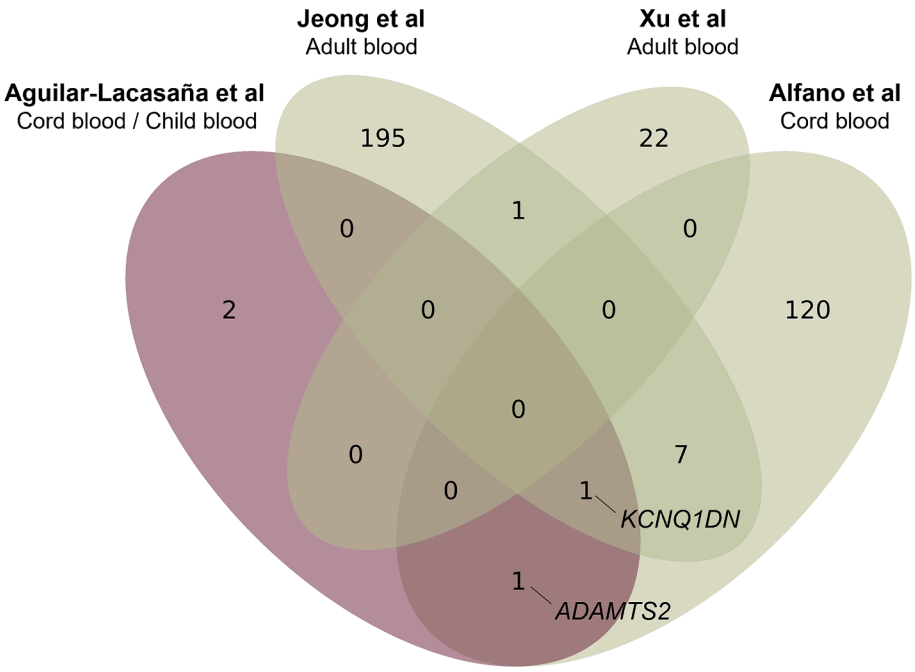

Fig. 3. Venn diagram showing the overlap of genes annotated to differentially methylated regions (DMR) in this study (Aguilar-Lacasaña et al.,) with previous studies (Alfano et al., 2023; Jeong et al., 2022; Xu et al., 2021b). There is overlap in population between the current study and Alfano et al.

influence health by reducing harm, decrease stress and increase attention, while NDVI within a buffer of 300 m may also influence health by promoting physical activity and social contacts, similar to residential proximity to a green space (Markevych et al., 2017). Half of the DMRs found in this study were associated with NDVI 100 m while the other half with NDVI 300 m. Two cord blood CpGs (cg06823681, cg14093792) within the DMR annotated to *SLC6A12* associated to NDVI 300 m have been previously linked to pre-pregnancy maternal body mass index in cord blood in cell composition unadjusted models. However, these associations were no longer significant after adjusting for cell composition (Sharp et al., 2017). Finally, we did not find any overlap between cord blood and child blood suggestive DMPs or DMRs, which might reflect different sample sizes and hence different statistical power.

One of the strengths of our study is the large sample size, which is three times greater than the largest previous EWAS of exposure to green space (Jeong et al., 2022). Another strength of the study is the availability of DNA methylation data in cord blood and child blood allowing the evaluation of associations with green space exposure in two periods: pregnancy and from pregnancy to childhood (cumulative exposure). Moreover, we used a standardised protocol for generating identical green space exposure variables in each cohort, and the analysis plan and methods were pre-specified by the leading team, although each cohort performed their own preferred quality control and normalization steps of the DNA methylation data. Results were validated through leave-one-out and sensitivity analyses. Neither air pollution or cellular composition seemed to mediate the effects of green space exposure on DNA methylation. A potential mediating role of temperature and relative humidity in the associations of exposure to green spaces with DNA methylation should be investigated in more detail, but initial findings in two of our studies did not suggest a major role. Other studies, however, have indicated that temperature per se, independent of greenness, is associated with DNA methylation (Xu et al., 2021a). Lastly, this is the first study using DataSHIELD (Gaye et al., 2014) for genome-wide DNA methylation analysis. This infrastructure provides a novel technological solution that can circumvent some of the challenges in facilitating the access of researchers to individual level data and also makes the analysis process more secure.

The study needs to be interpreted within the context of its limitations. First, we were not able to explore longitudinal changes in DNA methylation or persistent effects into childhood as the number of children for whom we had information on DNA methylation in cord- and child-blood was small. Second, whilst NDVI provides a standardised way to measure green space across different populations, it cannot differentiate vegetation types, which could be relevant for our analyses. Moreover, we did not have data on more sensitive measurements of greenness such as Enhanced Vegetation Index (EVI) or other important aspects of the green space exposure, such as whether any participants accessed the green space close to them and if so, the time spent and specific activities undertaken in a green space, quality characteristics of the green space or even the emotional responses elicited by these environments. Third, the study was restricted to white Europeans due to the lack of sufficient sample size for other ethnicities, thus we cannot generalize findings to other populations (Breeze et al., 2022a,b). Fourth, the current study did not examine sex-specific associations due to power limitations. Fifth, whilst we tried to adjust for confounders, like most EWAS we cannot assume that any of the associations we have found are causal, or that our largely null findings are influenced by masking confounding. In the same way, we cannot assume that differences in DNA methylation will affect health outcomes as epigenetic mechanisms are more complex that what can be discerned from DNA methylation (Min et al., 2021). In addition to being unable to explore associations with other epigenetic mechanisms, this study, in common with other EWAS, covers only a small proportion of the epigenome (i.e. ~ 2% of the 23 million CpGs for the 450K array) (Battram et al., 2022a). Finally, like most EWAS, we have explored associations with white blood cell DNA methylation in umbilical cord blood as a surrogate indicator of the offspring organs (Lin et al., 2017; Lurà et al., 2018) and child blood. However, it is crucial to investigate other relevant cells and tissues, such as the placenta that controls foetal development and consequently, if affected, it can have long term effects on health (Maccani & Marsit, 2009; Mortillo & Marsit, 2022).

5. Conclusions

Overall, we found little robust evidence of the association between green space exposure and blood DNA methylation. We did not find

associations between residential green space exposure and genome-wide DNA methylation levels in cord or child blood across 0.4 million CpGs. Although we identified associations between pregnancy and cumulative exposure to surrounding greenness with four DMRs, further studies are needed to validate the findings and provide additional insights in the underlying biological pathways.

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CRediT authorship contribution statement

Sofia Aguilar-Lacasaña: Conceptualization, Methodology, Software, Formal analysis, Data curation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Irene Fontes Marques:** Conceptualization, Methodology, Formal analysis, Writing - Review & Editing. **Montserrat de Castro:** Data curation, Methodology, Writing - Review & Editing. **Payam Dadvand:** Methodology, Writing - Review & Editing. **Xavier Escribà:** Software, Resources, Writing - Review & Editing. **Serena Fossati:** Data curation, Writing - Review & Editing. **Juan R González:** Software, Resources, Writing - Review & Editing. **Mark Nieuwenhuijsen:** Methodology, Writing - Review & Editing. **Rossella Alfano:** Formal analysis, Writing - Review & Editing, **Isabella Annesi-Maesano:** Resources, Funding acquisition, Writing - review & editing. **Sonia Brescianini:** Data curation, Writing - review & editing. **Kimberley Burrows:** Data curation, Writing - review & editing. **Lucinda Calas:** Data curation, Software, Writing - review & editing. **Ahmed Elhakeem:** Data curation, Writing - review & editing. **Barbara Heude:** Resources, Funding acquisition, Writing - review & editing. **Amy Hough:** Data curation, Writing - review & editing. **Elena Isaevska:** Formal analysis, Writing - Review & Editing. **Vincent W V Jaddoe:** Resources, Funding acquisition, Writing - Review & Editing. **Deborah A. Lawlor:** Methodology , Resources, Writing - Review & Editing. **Genevieve Monaghan:** Formal analysis, Data curation, Writing - review & editing. **Tim Nawrot:** Resources; Writing - Review & Editing. **Michelle Plusquin:** Resources; Writing - Review & Editing. **Lorenzo Richiardi:** Funding acquisition, Writing - review & editing. **Aidan Watmuff:** Software, Writing - Review & Editing. **Tiffany C Yang:** Data curation, Writing- review & editing. **Martine Vrijheid:** Conceptualization, Methodology, Funding acquisition,Writing - Review & Editing, Supervision. **Janine F Felix:** Conceptualization, Methodology, Writing - Review & Editing, Supervision. **Mariona Bustamante:** Conceptualization, Methodology, Writing - Review & Editing, Supervision.

Declaration of competing interest

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

Data availability

The authors do not have permission to share data.

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Ethics approval and consent to participate

All participating studies were approved by their local ethical committees.

Availability of data and materials

Genome-wide DNA methylation summarized results can be found at HELIX-omics webpage (https://helixomics.isglobal.org/) and Zenodo repository (https://doi.org/10.5281/zenodo.11058025). Individual cohort level data may be available by application to the relevant institutions after obtaining required approvals. Additional details and references to the study cohorts are available in Appendix B: Supplementary methods. The code for the analyses is available in this GitHub repository link: https://github.com/sofiaguilarl/EWAS_GreenSpace_Blood.git.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2024.108684.

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