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Residential greenness-related DNA methylation changes

Ayoung Jeong [a,b,*], Ikenna C. Eze [a,b], Danielle Vienneau [a,b], Kees de Hoogh [a,b], Dirk Keidel [a,b], Thomas Rothe [c], Luc Burdet [d], John W. Holloway [e], Debbie Jarvis [f,g], Florian Kronenberg [h], Gianfranco Lovison [a,b,i], Medea Imboden [a,b], Nicole Probst-Hensch [a,b,*]

[a] Swiss Tropical and Public Health Institute, Basel, Switzerland
[b] Department of Public Health, University of Basel, Switzerland
[c] Spital Davos, Davos, Switzerland
[d] Hôpital Intercantonal de la Broye, Payerne, Switzerland
[e] Human Development and Health, Faculty of Medicine, University of Southampton, UK
[f] MRC-PHE Centre for Environment and Health, Imperial College London, London, UK
[g] Population Health and Occupational Disease, National Heart and Lung Institute, Imperial College London, London, UK
[h] Institute of Genetic Epidemiology, Medical University of Innsbruck, Innsbruck, Austria
[i] Department of Economics, Business and Statistics, University of Palermo, Italy

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ABSTRACT

Background: Residential greenness has been associated with health benefits, but its biological mechanism is largely unknown. Investigation of greenness-related DNA methylation profiles can contribute to mechanistic understanding of the health benefits of residential greenness.
Objective: To identify DNA methylation profiles associated with greenness in the immediate surroundings of the residence.
Methods: We analyzed genome-wide DNA methylation in 1938 blood samples (982 participants) from the Swiss Cohort Study on Air Pollution and Lung and Heart Diseases in Adults (SAPALDIA). We estimated residential greenness based on normalized difference vegetation index at 30 × 30 m cell (green30) and 500 m buffer (green500) around the residential address. We conducted epigenome-wide association study (EWAS) to identify differentially methylated CpGs and regions, and enrichment tests by comparing to the CpGs that previous EWAS identified as associated with allergy, physical activity, and allostatic load-relevant biomarkers.
Results: We identified no genome-wide significant CpGs, but 163 and 56 differentially methylated regions for green30 and green500, respectively. Green30-related DNA methylation profiles showed enrichments in allergy, physical activity, and allostatic load, while green500-related methylation was enriched in allergy and allostatic load.
Conclusions: Residential greenness may have health impacts through allergic sensitization, stress coping, or behavioral changes. Exposure to more proximal greenness may be more health-relevant.

1. Introduction

Residential greenness has emerged as one of the determinants of the environmental burden of disease, with increasing evidence on its links with well-being and disease states. Health benefits of green areas in residential surroundings have been shown for general and mental health (Dadvand et al. 2016; Engemann et al. 2019; McEachan et al. 2016), sleep duration and quality (Astell-Burt et al. 2013; Grigsby-Toussaint et al. 2015), cognitive function (de Keijzer et al. 2018; Zhu et al. 2019), birth weight (Dzhambov et al. 2014), adiposity (Lovasi et al. 2013; Sarkar 2017), and all-cause mortality (Rojas-Rueda et al. 2019; Vienneau et al. 2017).

The mechanisms underlying the health benefits of greenness are not yet understood, but are thought to act via reduction of psychosomatic stress (Engemann et al. 2019; Hartig et al. 2014; Van den Berg et al. 2015), promotion of physical activity (Bancroft et al. 2015; Hooper et al.

* Corresponding authors at: Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Socinstrasse57, 4051 Basel, Switzerland (A. Jeong). Head Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Socinstrasse 57, 4051 Basel, Switzerland (N. Probst-Hensch).
E-mail addresses: ayoung.jeong@swisstph.ch (A. Jeong), nicole.probst@swisstph.ch (N. Probst-Hensch).

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2020; James et al. 2016), improvements in immune function (Kuo 2015), and reduced allostatic load (Egorov et al. 2017; Egorov et al. 2020; Ribeiro et al. 2019b). Yet, our understanding of the biological mechanisms driving the reported associations of these environmental attributes to health outcomes remains limited (Dzhambov et al. 2020; Kuo 2015; Markevych et al. 2017).

DNA methylation provides an essential tool in investigating the mechanistic linkage of environmental exposures to disease as it influences the expression of downstream molecules that drive disease development or progression (Keil and Lein 2016; Meehan et al. 2018). No study to date has investigated the association of residential greenness on the DNA methylome, despite the plausibility that greenness could influence DNA methylation and downstream health outcomes. First, greenness, in part, influences health via interactions with physical and chemical environmental factors that have been shown to influence DNA methylation (Alfano et al. 2018; Commodore et al. 2018; Guo et al. 2017; Rider and Carlsten 2019). Second, neighborhood deprivation, which often correlates negatively with greenness in urban areas (Astell-Burt et al. 2014), has also been reported to influence DNA methylation in recent studies (Giurgescu et al. 2019; Smith et al. 2017). Third, DNA methylation changes have been linked to physical activity (Fernández-Sanlés et al. 2020), allergy (Peng et al. 2019), and immune responses (Mendelson et al. 2018), which constitute potential pathways linking greenness to health. Therefore, investigating the link of greenness to DNA methylation could contribute to the much-needed understanding of the biological mechanisms linking greenness to downstream phenotypes, as well as identify shared or unique pathways in comparison to co-occurring and correlated exposures.

In this study we aimed to identify DNA methylation profiles associated with greenness in the immediate surroundings of the residence. While agnostic genome-wide investigation of DNA methylation offers the chance to identify novel biomarkers, the biological relevance of such results are often not straightforward. Changes in DNA methylation may or may not have regulatory functions on gene expression. As an attempt to provide more meaningful biological interpretation of the EWAS results, we curated candidate pathways (including allergy, physical activity and allostatic load, which capture the hypothesized greenness mechanisms) based on previously published EWAS, and tested enrichment of our EWAS results in these pathways.

2. Methods

2.1. Study population

The epigenetic analysis was conducted in SAPALDIA (Swiss Cohort Study on Air Pollution and Lung and Heart Diseases in Adults) in the context of the ALEC (Aging Lungs in European Cohorts) study. The protocol of SAPALDIA has been described previously (Ackermann-Liebrich et al. 2005; Martin et al. 1997). In brief, SAPALDIA was initiated in 1991 recruiting 9651 adults from eight areas representing diverse geography, meteorology, and urbanization in Switzerland. Out of the 9651 participants, 8047 and 6088 were followed-up in 2001–2003 (SAPALDIA2) and 2010–2011 (SAPALDIA3), respectively. Blood samples were collected and biobanked (−80 °C) at SAPALDIA2 and SAPALDIA3. In the context of the ALEC study, repeated blood samples at SAPALDIA2 and SAPALDIA3 from 987 representative participants (contributing 1974 samples) were analyzed for genome-wide DNA methylation (Imboden et al. 2019). This study investigated 982 participants (contributing 1938 samples) with full information on greenness and all covariates as well as DNA methylome available either at SAPALDIA2 or SAPALDIA3 or at both. Table 1 summarizes the study sample characteristics. SAPALDIA study protocol was approved by the Swiss Academy of Medical Sciences and the regional committees of the participating cantons. All participants provided written informed consent at enrolment.

Table 1
Study sample characteristics.

	SAPALDIA2	SAPALDIA3
N	972	966
Age [years]	50.2 (17.8)	58.5 (17.6)
Female	521 (53.6)	518 (53.6)
Education		
Low	54 (5.6)	54 (5.6)
Middle	634 (65.2)	634 (65.6)
High	284 (29.2)	278 (28.8)
Neighborhood SEP index [%]	64.4 (13.2)	64.4 (13.2)
Smoking status		
Never	402 (41.4)	394 (40.8)
Former	294 (30.2)	355 (36.7)
Current	276 (28.4)	217 (22.5)
Pack-years smoked	2.0 (18.4)	2.5 (21.1)
Second-hand smoking	257 (26.4)	136 (14.1)
Green30	0.583 (0.292)	0.592 (0.275)
Green500	0.588 (0.161)	0.594 (0.161)
Pearson correlation between Green30 and Green500	0.64	0.62
$PM_{2.5}$ [μg/m^3]	14.4 (4.7)	14.5 (1.5)
NO_2 [μg/m^3]	20.4 (14.3)	16.7 (8.9)
Aircraft Lden [dB]	30.0 (9.7)	30.5 (8.3)
Railway Lden [dB]	30.4 (11.0)	30.0 (7.2)
Road traffic Lden [dB]	53.8 (10.9)	54.0 (11.0)

Data are presented as count (%) or median (interquartile range). Green30: mean normalized difference vegetation index (NDVI) of the 30 × 30 m cell in which participant's home address was located; Green500: mean NDVI of 500 m circular buffer around participant's home address with the 30 × 30 m cell at the center excluded; NO_2: nitrogen dioxide; $PM_{2.5}$: particulate matter with aerodynamic diameter < 2.5 μm; SEP: socio-economic position.

2.2. DNA methylation

Genome-wide DNA methylation was measured in the repeated peripheral blood samples, using Illumina Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA) following manufacturer's protocol. Repeated blood samples were arranged across the arrays to minimize batch effect. DNA methylation data were processed using R package "minfi" (Aryee et al. 2014). Samples with sex mismatch were excluded. After background and dye-bias was corrected using Noob (normal-exponential out-of-band) procedure (Triche et al. 2013), β–values were computed as the ratio of methylated intensity over total intensity with offset 100. β–values with detection $p > 10^{-16}$ were set to missing. Probes with call rate < 0.95 were excluded. Samples with call rate < 0.95 were prespecified to be excluded but none of the samples had a call rate < 0.95. Illumina probe design bias was corrected by applying beta-mixture quantile normalization (BMIQ) (Teschendorff et al. 2013). The probes previously reported as non-specific or targeting polymorphic sites were excluded (Chen et al. 2013). We only used autosomal chromosome probes in this study. 433,741 CpGs were examined in this study.

2.3. Greenness estimates

Normalized difference vegetation index (NDVI) during summer was calculated from cloud- and snow-free satellite images covering the entire surface of Switzerland in 2014 (Vienneau et al. 2017). Residential greenness was assessed in two different metrics to represent immediate surrounding and local neighborhood separately. "Green30" was defined as the mean NDVI of the 30 × 30 m cell in which the participant's home address was located. "Green500" was defined as the mean NDVI of 500 m circular "buffer" (using focal functions in ArcGIS) around participant's home address with the 30 × 30 m cell at the center excluded (donut-shaped), to ensure independence of the two residential greenness metrics.

2.4. Covariates

We a priori considered a set of covariates as confounders of the association between greenness and DNA methylation. These included age (years), sex (male/female), study area (Basel/Wald/Davos/Lugano/Montana/Payerne/Aarau/Geneva), education level (primary school ("low"); secondary/middle school or apprenticeship ("middle"); college or university ("high")), neighborhood index of socio-economic position (SEP) (%) (derived from a principal component analysis of education, occupation, and housing variables from Switzerland census in 2000 (Panczak et al. 2012)), smoking status (never/former/current), pack-years smoked (calculated from self-reported information on cigarettes smoked per day and smoking history), second-hand smoke exposure (yes/no), residential exposure to particulate matter with aerodynamic diameter < 2.5 μm ($PM_{2.5}$) (annual mean exposure derived from PolluMap, national air pollution dispersion models for Switzerland in 2000 and 2010 (FOEN 2013)), residential exposure to nitrogen dioxide (NO_2) (annual mean exposure derived from land use regression models in 2000 (Liu et al. 2012) and biennial mean exposure derived from land use regression models in 2010–2011 (Eeftens et al. 2016)), residential exposure to transportation noise (annual average day-evening-night noise from aircraft, railway, or road traffic (Karipidis et al. 2014)), and season of the blood draw (Spring/Summer/Autumn/Winter). In addition, leukocyte composition (B cells, CD4+ T cells, CD8+ T cells, monocytes, natural killer cells, neutrophils, and eosinophils) estimated from DNA methylation using "estimateCellcounts" function implemented in the "minfi" package (Aryee et al. 2014) was included to prevent EWAS findings from capturing leukocyte composition changes instead of greenness-induced DNA methylation changes.

2.5. Statistical analysis

Fig. 1 describes the analysis scheme. Genome-wide DNA methylation data was measured in SAPALDIA. Data from the public repository of EWAS results (EWAS Atlas) (Li et al. 2019) and expression quantitative trait methylation (eQTM) data identified from the biobank-based integrative omics studies (BIOS) (Bonder et al. 2016) was used to interpret the findings from the SAPALDIA DNA methylome analysis.

2.5.1. EWAS

β-values were first regressed on 30 principal components derived from principal component analysis on 220 control probes incorporated on the Illumina 450 k array (Lehne et al. 2015). The residuals, presumably free from technical bias, were used as the DNA methylation values throughout the EWAS and referred to as DNAme in this manuscript. To minimize the effect of extreme values of DNAme (defined as 3 interquartile range (IQR) above or below the IQR for each probe) on the linear regressions, we identified these extreme values and replaced them with the corresponding threshold values for each probe. For 95% of all CpGs, we identified and replaced extreme values from less than 5% of the 1938 samples. DNAme was then regressed on both green30 and green500, after adjustment for the covariates listed above. We applied mixed models to address repeated measurement with a random intercept assigned to each participant. CpGs for which DNAme showed an association with greenness with Benjamini-Hochberg corrected p-value < 0.05 were declared genome-wide significant.

2.5.2. Differentially methylated regions (DMRs)

Given that DNA methylation patterns on neighboring CpGs often reflect regional changes in epigenetic state, we searched for DMRs using "DMRcate" R package (Peters et al. 2015). Gaussian kernel was used to transform z-statistics from EWAS into smoothed estimates. P-values were computed by comparing each point to the null distribution of the smoothed estimates. The regions containing at least one CpG with Benjamini-Hochberg corrected p-value < 0.05 defined DMRs.

2.5.3. Pathway enrichment tests of EWAS results

EWAS results are often translated at gene level by annotating CpGs to the nearest gene, which in turn are compared to pathway databases. However, DNA methylation does not always act through regulating nearest genes. In order to avoid this bias and to make the best use of the increasing EWAS literature, we curated pathways based on previously published EWAS (using data from the EWAS Atlas (Li et al. 2019)), and made direct comparison to our EWAS results. As most relevant for greenness exposure, we a priori considered allergy, physical activity, and allostatic load-related pathways. Search terms used to identify relevant EWAS results include: "rhinitis", "hay fever", "respiratory allergies", "atopy", "skin prick test", "allergic sensitization", "eczema", and "IgE" for allergy; "physical activity" and "exercise" for physical

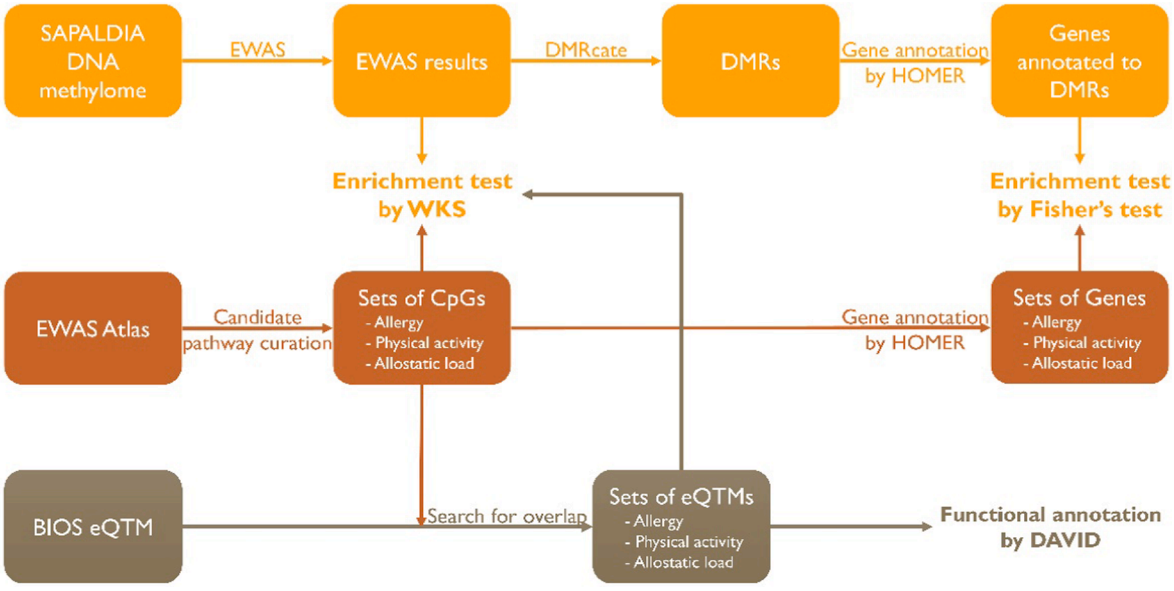

Fig. 1. Analysis scheme. BIOS: biobank-based integrative omics studies; DAVID: Database for annotation, visualization, and integrated discovery; DMRs: Differentially Methylated Regions; eQTM: expression quantitative trait methylation; EWAS: Epigenome-Wide Association Study; SAPALDIA: Swiss Cohort Study on Air Pollution and Lung and Heart Diseases in Adults; WKS: Weighted Kolmogorov-Smirnov test.

activity. We included EWAS results regardless of the tissue and the array used in the study, except we excluded one study on sperm. Allostatic load pathway was curated in a recent SAPALDIA study (Eze et al. 2020), where it proved to be relevant to traffic noise-related changes in DNA methylation profiles. We retrieved the CpGs with a p-value threshold < 1.1e-5 except for CRP-related CpGs which were identified with p-value < 1.15e-7 by additional literature search. Finally, "Allergy" pathway was curated as 3736 CpGs reported as associated with atopy or allergic sensitization. "Physical activity" pathway was curated as 361 CpGs reported as associated with physical activity or exercise. "Allostatic load" pathway consisted of multiple sub-pathways reflecting either immunological ("C-reactive protein (CRP)" as 258 CpGs associated with CRP), metabolic ("Metabolic syndrome" as 10 CpGs associated with metabolic syndrome; "Lipid" as 16 CpGs associated with blood triglycerides or high-density lipoprotein cholesterol; "Impaired fasting glucose (IFG)" as 9 CpGs associated with impaired fasting glucose or HbA1c; "Insulin" as 175 CpGs associated with insulin level or insulin resistance; "Central obesity" as 170 CpGs associated with abdominal obesity, waist circumference, or visceral adipose tissue; "Body mass index (BMI)" as 919 CpGs associated with BMI or general obesity), renal ("Kidney function" as 297 CpGs associated with estimated glomerular filtration rate or chronic kidney disease), and cardiovascular and autonomic nervous system ("Blood pressure" as 20 CpGs associated with systolic or diastolic blood pressure; "Cardiac autonomic function" as 8 CpGs associated with cardiac autonomic responses). The entirety of the 1675 CpGs in the 10 sub-pathways constituted "Allostatic load" pathway. The candidate pathways and their curation are summarized in Table 2 and supplementary Table S1. We applied the Weighted Kolmogorov-Smirnov test (Charmpi and Ycart 2015) to examine enrichments of the EWAS results in the candidate pathways. This algorithm produces null distribution by 10,000 Monte-Carlo simulations from the genome-wide z-statistics and compares the set of z-statistics mapped to each pathway with the null distribution. The pathway was declared enriched if Kolmogorov-Smirnov p-value < 0.05 after permutation-based multiple testing correction.

Table 2
Candidate pathways compared to BIOS eQTM.

Candidate pathway	#CpGs [a]	#eQTM[b] (%; Fisher's exact test p-value)	Top 3 KEGG pathways enriched for the genes annotated to the eQTM [c]
Allergy	3736	260 (7%; 4.1e-41)	Herpes simplex infection; Pertussis; Pathways in cancer
Physical activity	361	17 (5%; 0.021)	Antigen processing and presentation; Phagosome; Herpes simplex infection
Allostatic load	1675	231 (14%; 3.1e-90)	Antigen processing and presentation; Phagosome; Graft-versus-host disease
CRP	258	57 (22%; 1.5e-34)	TNF signaling pathway; Insulin signaling pathway
Metabolic syndrome	10	3 (30%; 0.0021)	N/A
Lipid	16	7 (44%; 1.0e-07)	Insulin resistance; AMPK signaling pathway
Impaired fasting glucose	9	4 (44%; 6.2e-05)	N/A
Insulin	175	7 (4%; 0.34)	Staphylococcus aureus infection; Complement and coagulation cascades; Pertussis
Central obesity	170	27 (16%; 2.2e-13)	Antigen processing and presentation; Graft-versus-host disease; Phagosome
BMI	919	147 (16%; 9.2e-67)	Antigen processing and presentation; Epstein-Barr virus infection; Herpes simplex infection
Kidney function	297	38 (13%; 5.0e-15)	N/A
Blood pressure	20	6 (30%; 1.1e-05)	N/A
Cardiac autonomic function	8	0 (0%; 1.00)	N/A

[a] Number of CpGs identified from EWAS catalog as associated with relevant phenotypes.
[b] Number of CpGs associated with the phenotype that overlap with eQTM identified by BIOS.
[c] DAVID enrichment test for the genes regulated by the eQTMs overlapping with the CpGs in each candidate pathway.

2.5.4. Pathway enrichment tests of DMRs

DMR analysis itself seeks a functional interpretation of the DNA methylation profiles, searching for a set of neighboring CpGs instead of a single CpG that is associated with the phenotype of interest. DMRs, however, do not necessarily constitute functional entities and they are often only annotated to the nearest genes. To derive more functional interpretation of the DMRs, we tested the same hypothesis in parallel to the EWAS results. All CpGs and DMRs were consistently annotated to genes with the closest transcription start site (TSS) using HOMER v4.11 (Heinz et al. 2010) so that the enrichment of the aforementioned candidate pathways could be examined at gene level. We applied Fisher's exact test to determine the gene-level enrichments. The pathway was declared enriched if Benjamini-Hochberg corrected p-value < 0.05.

2.5.5. Comparison to expression quantitative trait methylation (eQTM)

A challenge in interpretation of EWAS findings lies in the limited understanding of the regulatory functions of CpGs. Study of eQTMs can shed light on the effect of DNA methylation on gene expression. We took advantage of a public repository of *cis*-eQTM (+/− 250 kb) identified in the BIOS study (Bonder et al. 2016) (available from https://genenetwork.nl/biosqtlbrowser/) to understand the functional implications of greenness-related DNA methylation profiles. 12,809 *cis*-eQTMs with FDR < 0.05 were identified in whole blood samples from adult cohorts in BIOS by examining genome-wide DNA methylation and gene expression profiles from 2101 samples. First, we compared the CpGs in the candidate pathways with the BIOS eQTM. The genes regulated by the eQTMs overlapping with the CpGs in the candidate pathways were further interrogated for enrichments in KEGG pathways using Database for annotation, visualization, and integrated discovery (DAVID) (Dennis et al. 2003). Second, the Weighted Kolmogorov-Smirnov test of the EWAS results were repeated after restriction to pathway CpGs that were also eQTMs in the BIOS dataset.

2.6. Role of the funding source

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3. Results

3.1. EWAS and DMR analysis

EWAS found no genome-wide significant associations but seven and nine non-overlapping suggestive signals with p < 1e-05 for green30 and green500 respectively. All the EWAS signals with p < 1e-04 are listed in the supplementary Tables S2 and S3. We identified 163 and 56 DMRs for green30 and green500, respectively (supplementary Tables S4 and S5). The DMRs for green30 and green500 largely differed with only 6 DMRs identified for both green30 and green500. Approximately half of the DMRs are located in promoter region or exon of a protein-coding gene (74 out of 163 DMRs for green30 and 30 out of 56 for green500).

3.2. Pathway enrichments

We tested enrichments of the candidate pathways of our own curation for both EWAS results and DMRs. Comparison was at the CpG level for EWAS results and at the gene level for DMRs, after both the CpGs in the candidate pathways and DMRs were annotated to the genes with nearest TSS.

"Allergy" was consistently enriched for both green30 and green500 in both EWAS results and DMRs. "Physical activity" was enriched for green30 in both EWAS and DMRs, but not for green500. "Allostatic load" showed enrichment for green30 DMRs and also for green500 EWAS and DMRs (Table 3). The sub-pathways constituting "Allostatic load" showed sporadic enrichment (Table S6). "CRP", "BMI", and "Cardiac autonomic function" were enriched only for green30 DMRs and "Metabolic syndrome" only for green30 EWAS results. "Insulin" and "Kidney function" were enriched only for green500 DMRs.

3.3. Comparison to BIOS eQTM

All the candidate pathways examined in this study showed over-representation of eQTM except for "Insulin" and "Cardiac autonomic function" (Table 2). We observed highest overlap with eQTM in "Lipid" (7 out of 16) and "Impaired fasting glucose" (4 out of 9).

The genes regulated by the overlapping eQTM in the candidate pathways often showed over-representation in infectious diseases or immune related KEGG pathways (Table 2; for the full DAVID results see supplementary Tables S7-S14). When the pathway enrichment test of EWAS results was restricted to the eQTM, however, no pathway showed enrichment except "CRP" in green500 (Table 4 and S15).

4. Discussion

Green30 and green500 differ in their association with DNA methylation pattern, despite the high degree of correlation between these exposures (Pearson correlation coefficient > 0.6; see Table 1). Only a small fraction of the associated CpGs and DMRs in the EWAS for green30 and green500 overlapped (1/56 CpGs and 6/163 DMRs overlapped; see Table S2-S5). Green30 showed stronger evidence of enrichment (smaller p-values) than green500 consistently across all candidate pathways, regardless of being tested in EWAS results or DMRs, except for "Allostatic load" in EWAS results.

Allergy has been associated with greenness exposure, presumably via sensitization to allergens produced by specific vegetation within green spaces (Fuertes et al. 2016). Therefore, we hypothesized that the greenness-related DNA methylation pattern would coincide with allergy-related DNA methylation pattern. Our findings confirmed this hypothesis, although by design this study cannot determine whether greenness exposure increases or decreases allergic sensitization. Although pollen may travel very long distance, the amount of transported pollen decreases rapidly with increasing distance (Chamecki et al. 2009). Our findings of "Allergy" enrichment stronger for green30 than for green500 may attribute to proximal greenness leading to higher pollen load in the air.

Greenness may have health impact through changes in physical activity. Greener neighborhood may invite people to be more active (Bancroft et al. 2015; James et al. 2016). In this study, green30 showed enrichment for "Physical activity" but green500 did not. Consistently, "BMI" was also enriched for green30 DMRs but not for green500. This should in principle validate the enrichment of physical activity, provided that greenness-associated physical activity led to changes in BMI. Bancroft and colleagues reported in their systematic review that smaller buffers predicted objectively measured physical activity better than larger buffers, although studied buffer sizes usually ranged from 100 m to 1000 m (Bancroft et al. 2015). Greenness in immediate surrounding may differ from greenness in local neighborhood in its nature and hence influence people's behavior differently. However, we cannot rule out the possibility that our finding is due to residual confounding by socioeconomic position, i.e. wealthier people are exposed to higher greenness in their proximal environment and tend to be more physically active. Analyzing NDVI does not inform us about access or proximity to green spaces, which could be more relevant to investigate physical activity. Longitudinal studies with detailed information on use of different types of greenspace and on different types of physical activity and possibly taking moving into consideration are warranted to investigate which types of greenness actually motivates different types of physical activity and in which mechanism.

Allostatic load conceptualizes physiological "wear and tear" from repeated response to stressors, which has been associated with greenness (Egorov et al. 2017; Egorov et al. 2020; Ribeiro et al. 2019b), socioeconomic status (Johnson et al. 2017; Ribeiro et al. 2019a), and mortality (Castagne et al. 2018; Robertson et al. 2017). Our findings are in line with the hypothesis that greenness may help coping with stress. "Allostatic load", but none of its sub-pathways, showed consistent enrichment for green30 and green500, indicating that the enrichment of "Allostatic load" was not driven by a specific phenotype component. A recent review that discussed the lack of established definition of allostatic load concluded that despite the heterogeneity of the definition, the composite score of allostatic load consistently demonstrated association with detrimental health outcomes (Johnson et al. 2017). We recently demonstrated, using the same curation, that "Allostatic load" was enriched for traffic noise-related DNA methylation profiles (Eze et al. 2020).

Comparison with the BIOS eQTM demonstrated that the eQTM are over-represented in the CpGs of most of the candidate pathways curated in this study, indicating that the DNA methylation profiles reported by previous EWAS studies, and our curation thereof, likely have regulatory implications.

When the pathway enrichment tests were limited to eQTM in the candidate pathways, we lost all enrichments except CPR for green500. Weighted Kolmogorov-Smirnov takes advantage of making use of the genome-wide z-statistics to create a null distribution. By limiting to the eQTM, the null distribution was computed based on the EWAS results on 13 k eQTMs instead of 450 k CpGs, which may not serve as the best null distribution, or reduce the power, to determine the enrichment.

As for all bioinformatics research, this study depends on the quality and quantity of previous research. Unlike genomics and transcriptomics, research of genome-wide DNA methylation has a relatively short history and studies conducted so far are typically smaller in number and size. The credibility of pathway curation based on EWAS Atlas varies across phenotypes. It may work better for well-studied phenotypes, e.g. BMI,

Table 3
Pathway enrichment p-values from Weighted Kolmogorov-Smirnov test of EWAS results and from Fisher's exact test of DMRs.

		Allergy	Physical activity	Allostatic load
#CpGs		3736	361	1675
Green30	EWAS	**0.00053**	**0.0016**	0.15
	DMR	**3.1e-06**	**4.6e-05**	**2.6e-05**
Green500	EWAS	**0.0021**	0.22	**0.0069**
	DMR	**0.023**	1.00	**0.024**

Written in bold if multiple testing adjusted $p < 0.05$.

Table 4
Pathway enrichment p-values from Weighted Kolmogorov-Smirnov test of EWAS results restricted to eQTM.

	Allergy	Physical activity	Allostatic load
#CpGs	260	17	231
Green30	0.15	0.66	0.42
Green500	0.10	0.52	0.76

Written in bold if multiple testing adjusted $p < 0.05$.

than for less well-studied phenotypes, e.g. cardiac autonomic function. The curation of allostatic load pathway was limited to the availability of phenotypes whose DNA methylation pattern was studied and reported. "Allostatic load" pathway in this study did not include the hypothalamic–pituitary-adrenal axis as there has been no EWAS for relevant phenotypes. We attempted to explore functionality of EWAS results making use of BIOS eQTM, which allows more direct regulatory interpretation than proximity-based gene annotation. However, BIOS only investigated *cis*-eQTMs, although these were in adult whole blood matching the tissue source of DNAme in SAPALDIA. Large comprehensive future studies to search for *trans*-eQTMs are warranted. By using NDVI as greenness metric, we cannot distinguish different types of greenness, nor evaluate whether they contain potential allergens or are conducive to physical activity. Also, given the cross-sectional nature of the study we cannot decide whether the presence of greenness leads to the observed phenotypes or whether for example people with allergies and high levels of stress choose greener environments to live in. Lastly, we estimated the residential greenness based on the home address reported at the time of surveys without taking into account time spent at home, secondary residential addresses, or greenness exposure at work. However, we believe such measurement errors are non-differential and therefore bias towards null.

5. Conclusions

In this study we explored genome-wide DNA methylation patterns related to residential greenness. We identified largely non-overlapping 163 and 56 DMRs for green30 and green500 respectively. Green30 showed consistently stronger enrichment than green500 across candidate pathways curated based on previously reported EWAS. Residential greenness may have health impacts through allergic sensitization, stress coping, or behavioral changes.

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CRediT authorship contribution statement

Ayoung Jeong: Conceptualization, Data curation, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Ikenna C. Eze:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Danielle Vienneau:** Data curation, Investigation, Writing – review & editing. **Kees de Hoogh:** Data curation, Investigation, Writing – review & editing. **Dirk Keidel:** Data curation, Investigation, Project administration, Writing – review & editing. **Thomas Rothe:** Project administration, Writing – review & editing. **Luc Burdet:** Project administration, Writing – review & editing. **John W. Holloway:** Methodology, Investigation, Writing – review & editing. **Debbie Jarvis:** Methodology, Investigation, Writing – review & editing. **Florian Kronenberg:** Investigation, Writing – review & editing. **Gianfranco Lovison:** Conceptualization, Methodology, Writing – review & editing. **Medea Imboden:** Conceptualization, Project administration, Writing – review & editing. **Nicole Probst-Hensch:** Conceptualization, Methodology, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

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Study directorate: NM Probst-Hensch (PI; e/g); T Rochat (p), C Schindler (s), N Künzli (e/exp), JM Gaspoz (c)

Scientific team: JC Barthélémy (c), W Berger (g), R Bettschart (p), A Bircher (a), C Brombach (n), PO Bridevaux (p), L Burdet (p), Felber Dietrich D (e), M Frey (p), U Frey (pd), MW Gerbase (p), D Gold (e), E de Groot (c), W Karrer (p), F Kronenberg (g), B Martin (pa), A Mehta (e), D Miedinger (o), M Pons (p), F Roche (c), T Rothe (p), P Schmid-Grendelmeyer (a), D Stolz (p), A Schmidt-Trucksäss (pa), J Schwartz (e), A Turk (p), A von Eckardstein (cc), E Zemp Stutz (e).

Scientific team at coordinating centers: M Adam (e), I Aguilera (exp), S Brunner (s), D Carballo (c), S Caviezel (pa), I Curjuric (e), A Di Pascale (s), J Dratva (e), R Ducret (s), E Dupuis Lozeron (s), M Eeftens (exp), I Eze (e), E Fischer (g), M Foraster (e), M Germond (s), L Grize (s), S Hansen (e), A Hensel (s), M Imboden (g), A Ineichen (exp), A Jeong (g), D Keidel (s), A Kumar (g), N Maire (s), A Mehta (e), R Meier (exp), E Schaffner (s), T Schikowski (e), M Tsai (exp)

(a) allergology, (c) cardiology, (cc) clinical chemistry, (e) epidemiology, (exp) exposure, (g) genetic and molecular biology, (m) meteorology, (n) nutrition, (o) occupational health, (p) pneumology, (pa) physical activity, (pd) pediatrics, (s) statistics

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106945.

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