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Higher buccal mtDNA content is associated with residential surrounding green in a panel study of primary school children

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A R T I C L E I N F O

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A B S T R A C T

Background: Mitochondria are known to respond to environmental stressors but whether green space is associated with mitochondrial abundance is unexplored. Furthermore, as exposures may affect health from early life onwards, we here evaluate if residential green space is associated with mitochondria DNA content (mtDNAc) in children.

Methods: In primary schoolchildren (COGNAC study), between 2012 and 2014, buccal mtDNAc was repeatedly (three times) assessed using qPCR. Surrounding low (<3m), high (≥3m) and total (sum of low and high) green space within different radii (100m–1000m) from the residence and distance to the nearest large green space (>0.5ha) were estimated using a remote sensing derived map. Given the repeated measures design, we applied a mixed-effects model with school and subject as random effect while adjusting for *a priori* chosen fixed covariates.

Results: mtDNAc was assessed in 246 children with a total of 436 measurements (mean age 10.3 years). Within a 1000m radius around the residential address, an IQR increment in low (11.0%), high (9.5%), and total (13.9%) green space was associated with a respectively 15.2% (95% CI: 7.2%–23.7%), 10.8% (95% CI: 4.5%–17.5%), and 13.4% (95% CI: 7.4%–19.7%) higher mtDNAc. Conversely, an IQR increment (11.6%) in agricultural area in the same radius was associated with a −3.4% (95% CI: 6.7% to −0.1%) lower mtDNAc. Finally, a doubling in distance to large green space was associated with a −5.2% (95% CI: 7.9 to −2.4%) lower mtDNAc.

Conclusion: To our knowledge, this is the first study evaluating associations between residential surrounding green space and mtDNAc in children. Our results showed that green space was associated with a higher mtDNAc in children, which indicates the importance of the early life environment. To what extent these findings contribute to later life health effects should be further examined.

1. Introduction

Mitochondria are essential for the function and homeostasis of the cell due to their role in energy (ATP) production, apoptosis, the control of cytosolic calcium levels, and cell signalling (Shaughnessy et al., 2014). They have their own double-stranded genome, called mitochondrial DNA. Being the powerhouse of the cell, mitochondrial DNA content (mtDNAc) in cells and tissues must be adjusted according to the metabolic need (Filograna et al., 2021). As a proxy for mitochondrial function (Malik and Czajka, 2013), mtDNAc is used to assess the role of mitochondria in disease (Longchamps et al., 2020). As such, a higher mtDNAc (measured in peripheral blood, buccal cells and placental tissue, respectively) has been associated with cancer (Zhou et al., 2014) in adults and heart rate variability (Saenen et al., 2019) and intelligence (Bijnens et al., 2019) in children. On the other hand, a lower mtDNAc (measured in peripheral blood and leucocytes, respectively) has been

Abbreviations: Cq, cycle quantification value; mtDNAc, mitochondrial DNA content; IQR, interquartile range; GIS, Geographic Information System.

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associated with health outcomes such as Parkinson's disease (Pyle et al., 2016), and schizophrenia (Shivakumar et al., 2020) in adults.

Literature suggests that environmental exposures might affect mtDNAc as mitochondria coordinate cellular adaptation to stressors like oxidative stress and DNA damage (Vyas et al., 2016). In adults, a decreased mtDNAc has been associated with particulate matter air pollution (Wang et al., 2020) and PAH exposure (Pavanello et al., 2013). Although limited, this effect is also observed during childhood. For example, exposure to particulate matter has been associated with a lower buccal mtDNAc (Hautekiet et al., 2020; Saenen et al., 2019) and exposure to lead was associated with a lower leukocyte mtDNAc (Alegría-Torres et al., 2020). What has, to our knowledge, not been evaluated in literature is the effect of green space on mtDNAc. Assessing green space in early life is important as this might affect health outcomes later in life. For example, reduced exposure to residential green space during early life has been associated with higher blood pressure during adulthood (Bijnens et al., 2017). Also, children living in the lowest decile of green space showed a 15%–55% higher risk for mental illnesses later in life compared to those living in the highest decile of green space (Engemann et al., 2019). Hence, in this panel study, we examined whether residential surrounding green space and distance to green space were associated with mtDNAc in children between 9 and 12 years old. We hypothesize that children living in a neighbourhood with more green space or living close to green space have higher mtDNAc.

2. Materials and methods

2.1. Study population

This study was part of the COGNAC (COGNition and Air pollution in Children) study and included repeated measurements of children between 9 and 12 years old (Saenen et al., 2016). The study was executed between January 2012 and February 2014 in three different schools, each in another Belgian municipality (Tienen, Zonhoven, and Hasselt-Kiewit). 770 children were invited, of which 334 (43.4%) agreed to participate. In the present study, a final study population of 246 children (73.7%), with a total of 436 measurements were included after exclusion of samples not meeting the quality control of the molecular measurements.

Parents of the children signed an informed consent and completed a questionnaire to provide information on the demographic and lifestyle characteristics of the child and its family. Socioeconomic status was based on the highest educational level of either parent (up to high school diploma; college or university diploma). Children were considered to be exposed to passive smoking if one or more family members smoked inside the house. Physical activity like for example soccer, basketball, gymnastics, swimming, or horseback riding was calculated in hours per week. The study complied with the Helsinki declaration and was approved by the ethics committees of Hasselt University and the Eastern-Limburg Hospital, Belgium (11/065U).

2.2. Sample collection

Buccal cell swabs (SK2, Isohelix, Kent, UK) were sampled during three different examinations at the school. Per examination, we collected two buccal samples per child. Sampling for each examination was carried out at the same time during the day and on the same day of the week. Before sampling, the children did not eat or drink for at least 30 min and rinsed their mouths three times with water. Each swab was rubbed inside the cheek for 1 min. Afterwards the samples were kept on ice until storage at −80 °C. The mean (SD) time interval between two consecutive examinations was 46 (20) days. Of the 246 children included, 105 (42.7%) provided a buccal swab at one time point, 92 (37.4%) at two time points, and 49 (19.9%) at three time points, resulting in a total number of 436 samples.

2.3. Mitochondrial DNA assay

DNA was extracted from both buccal swabs at once using the QIAgen Micro Kit (Qiagen, N.V. Venlo, the Netherlands). The quantity and purity of the sample were measured with a Nanodrop spectrophotometer (ND-1000; Ismgen Life Science, De Meern, the Netherlands). To ensure a uniform DNA input for each qPCR reaction, samples were diluted and checked using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Europe). Extracted DNA was stored at −80 °C until analysis.

Average mtDNAc was determined by averaging the ratios of two mitochondrial gene copy numbers (MTF3212/R3319 and MT-ND1) (M) to one reference gene (36B4) (S). Samples were measured in triplicate using a previously described quantitative real-time PCR (qPCR) assay with minor modifications (Janssen et al., 2012). Briefly, the reaction mixtures contained Qiagen 1x QuantiTect SYBR Green Mastermix, forward (300 nM) and reverse (300 nM) primer, and 12.5 ng DNA. The forward and reverse primers for the mitochondrial genes were respectively 5′-CACCCAAGAACAGGGTTTGT-3′ and 5′-TGGCCATGGGTATGT TGTTAA-3′ for MTF3212/3319, and 5′-ATGGCCAACC TCCTACTCCT-3′ and 5′-CTACAACGTTGGGGCCTTT-3′ for MT-ND1. For the reference gene, the forward and reverse primers were 5′-GGAATGTGGGCTT TGTGTTC-3′ and 5′-CCCAATTGTCCCCTTACCTT-3′.

All PCR-reactions were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following thermal cycling profile: 'M' – 1 cycle of 10 min at 95 °C, followed by 30 cycles of 15 s at 95 °C and 1 min 10 s at 58 °C; 'S' – 1 cycle of 10 min at 95 °C, followed by 35 cycles of 15 s at 95 °C and 1 min 10 s at 58 °C. Six inter-run calibrators (IRCs) were used to account for inter-run variability. Also, non-template controls were used in each run. Raw data were processed and normalized to the reference gene using qBase-plus software (Biogazelle, Zwijnaarde, Belgium), taking into account run-to-run differences. Quality control measures were taken into account for data pre-processing after qPCR: (1) absolute difference <0.3 of triplicate Ct (cycle threshold) values and (2) maximum 1 absolute Ct value deviation of the average Ct of each plate. The intra-plate ICC (95% CI) of M/S ratios (MTF3212/3319 and MT-ND1), mtDNAc runs (MTF3212/3319 and MT-ND1) and single-copy gene runs were respectively 0.953 (0.945, 0.960), 0.962 (0.955, 0.968), 0.975 (0.970, 0.978), 0.957 (0.950, 0.963), and 0.975 (0.971, 0.979) (Telomere research network, 2020).

2.4. Residential green space exposure

Residential addresses of the children were geocoded. We characterized two indicators of exposure to green space: 1) residential surrounding green space (%) and 2) residential distance to green space (m). To characterize green space we used the Green Map of Flanders (2012), generated by the Agency for Geographic Information Flanders (AGIV) (Geopunt Vlaanderen 2012). This 1 m^2 resolution raster geodataset derived from orthorectified aerial imagery classifies every pixel in the entire region as: not green, agricultural area, low green (vegetation height <3 m), and high green (vegetation height ≥3 m) areas. The percentage of agricultural area and low, high, and total (sum of low and high green space) green space was calculated for different radii (100 m, 300 m, 500 m, and 1000 m) around each child's residence. To evaluate scale effects and the consistency and robustness or our results, we chose different radii. The 100 m, 300 m, and 500 m radius were used to indicate the immediate, intermediate, and neighbourhood surrounding green space, respectively, whereas 1000 m indicates the common walking distance to places nearby (Dadvand et al., 2017; Stockton et al., 2016). Finally, to characterize residential distance to green space, we calculated the distance (m) from the child's residence to the nearest large green space (≥0.5 ha) (Ludlow et al., 2003). For children with more than one residential address at the moment of the study (n = 38; 15.5%), we calculated a weighted average using the proportion of time spent at each location. ArcGIS 10 (ESRI Inc, Redmonds, CA, US) software was used for all GIS analyses.

2.5. Statistical analysis

Statistical analyses were performed using SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). To reduce skewness and to approximate a normal distribution, we log (10)-transformed the mtDNAc data, the distance to major roads, and the distance to green space. As our study design included repeated measurements of mtDNAc, we used a mixed-effects model (unstructured covariance matrix) with school (representing the sampling framework) and subject as random effects to assess the associations between surrounding green space exposure or distance to green space and mtDNAc as the outcome variable. *A priori* selected covariates included age (continuous), sex (boy; girl), BMI (continuous), passive smoking (yes; no), season of examination (winter; summer; autumn; spring), highest educational level of either parent (up to high school; college or university), date of examination (continuous) and distance to major road (continuous). Distance to major road represents the distance from the child's residence to the nearest highway or other national road and was calculated with ArcGIS 10 (ESRI Inc, Redmonds, CA, US) software. We chose these covariates as socio-economic status has been associated with greens space (P. Dadvand et al., 2012) whereas the others have been associated with mtDNAc (Hou et al., 2019; Janssen et al., 2012; Knez et al., 2016; Skuratovskaia et al., 2019; Wu et al., 2019).

All covariates were measured at baseline except season, which was accounted for at each examination. Results of the analyses were presented as the percentage difference in relative average mtDNAc associated with an interquartile range (IQR) increment in residential green space or with a doubling in distance to green space. Because of the skewedness of the data for the 100 m radius of agriculture, we expressed the effect size for a dichotomized variable comparing the presence to the absence of agriculture within this radius.

Bivariate associations between the fixed covariates and the outcome were assessed using the unadjusted model, but taking into account the random effect of the subjects and school. Unadjusted Spearman's correlations were used to assess the associations between the different types of green space within each of the radii.

2.6. Senstivity analysis

In a sensitivity analysis, we additionally adjusted the model separately and combined for residential $PM_{2.5}$ exposure in the year before examination (long-term exposure), the average ultrafine particle (UFP) < 300 nm exposure at school measured between 9 and 12 O'clock on the day of examination (short-term exposure), and physical activity, based on out-of-school sport activities (hours/week).

Daily residential exposure (μg/m^3) to particulate matter ≤2.5 μm ($PM_{2.5}$) at the child's residence was modelled using a spatiotemporal interpolation model, designed for Belgium (Janssen et al., 2008; Lefebvre et al., 2013; Maiheu et al., 2013), as described before (Hautekiet et al., 2020). We used the daily modelled exposure values obtained by this model to assign the residential exposure during the year before each examination. For children with more than one residential address at the moment of the study, we calculated a weighted average using the proportion of time spent at each location. Exposure to ultrafine particles (UFP) < 300 nm at school was measured continuously between 9 and 12 O'clock with a nanotracer (Aerasense, Best, The Netherlands) on the day of the examination.

Finally, to evaluate the effect of the repeated measures design, we also made the association between green space and mtDNAc when only the first measurement of each child was included. Mixed models as for the main analysis were used but without the random statement.

3. Results

3.1. Population characteristics

The characteristics of the 251 children included in this study are presented in Table 1. Children comprising of 46.8% girls had an average age and BMI (SD) of 10.3 (1.3) years and 17.4 (2.8) kg/m^2, respectively. The most common highest category of education of either parent was college or university (69.5%). 15.9% of the children were exposed to passive smoking at home. The average distance (SD) to major road was 576.2 (559.0) m. The children's geometric mean of relative mtDNAc (95% CI) was 0.98 (0.94–1.01). The mtDNAc results of the three examinations were positively correlated with each other: the Pearson correlation coefficients between examinations 1 and 2, 1 and 3, and 2 and 3 were respectively 0.44 ($p < 0.0001$), 0.30 ($p = 0.018$), and 0.27 ($p = 0.023$) (Suppl. figure 1). The average number of days between examination 1 and 2, 1 and 3 and 2 and 3 was respectively 55, 95 and 27 days.

Bivariate analysis showed that girls tended to have a −6.8% (95% CI: 14.2% to 1.3%, $p = 0.095$) lower mtDNAc compared to boys. A one-year increase in age was associated with a 3.9% (95% CI: 0.5 to 7.3, $p = 0.023$) higher mtDNAc. No significant association was found between mtDNAc and BMI ($p = 0.57$), exposure to passive smoking ($p = 0.16$), education ($p = 0.54$), or physical activity ($p = 0.80$).

3.2. Green space exposure characteristics

Mean exposure (IQR) to low green, high green, total green and agriculture in a 1000 m radius was respectively 28.7 (11.0)%, 20.9 (9.5)%, 49.6 (13.9)%, and 17.0 (13.9)% (Table 2). The median distance (IQR) to large green space was 123.7 (144.6) m (Table 2). The different types of green space and the different radii were highly correlated (Suppl. Table 1). Agricultural area was negatively correlated with low and total green, with the strongest association between low green and agriculture in a 1000 m radius (Spearman's correlation = −0.54, p < 0.001).

3.3. Residential surrounding green space and distance to green space in association with mtDNAc

After adjustment for the *a priori* selected covariates, residential surrounding green space was positively associated with mtDNAc in all buffers except for the 100 m radius (Fig. 1 & Suppl. Table 2). The strongest associations were found for the 500 m and 1000 m radii. An IQR increment in low (11.0%), high (9.5%), and total (13.9%) green space in the 1000 m radius was associated with respectively a 15.2% (95% CI: 7.2% to 23.7%, $p = 0.00014$), 10.8% (95% CI: 4.5% to 17.5%, $p = 0.00070$), and 13.4% (95% CI: 7.4% to 19.7%, $p < 0.0001$) higher mtDNAc (Fig. 1). On the contrary, the opposite effect was found for

Table 1
Characteristics of the study population (n = 246).

Characteristics	Mean (SD) or n (%)
Girls	115 (46.8%)
Age, years	10.3 (1.3)
Body Mass Index (BMI) (kg/m^2)	17.4 (2.8)
Highest category of education of either parent	
Up to high school diploma	75 (30.5%)
College or university diploma	171 (69.5%)
Exposed to passive tobacco smoke	39 (15.9%)
School	
Tienen	56 (22.8%)
Zonhoven	122 (49.6%)
Hasselt-Kiewit	68 (27.6%)
Distance to major road (m)	576.2 (559.0)
Physical activity (hours/week) [a]	3.4 (2.4)

[a] Data available for 240 children.

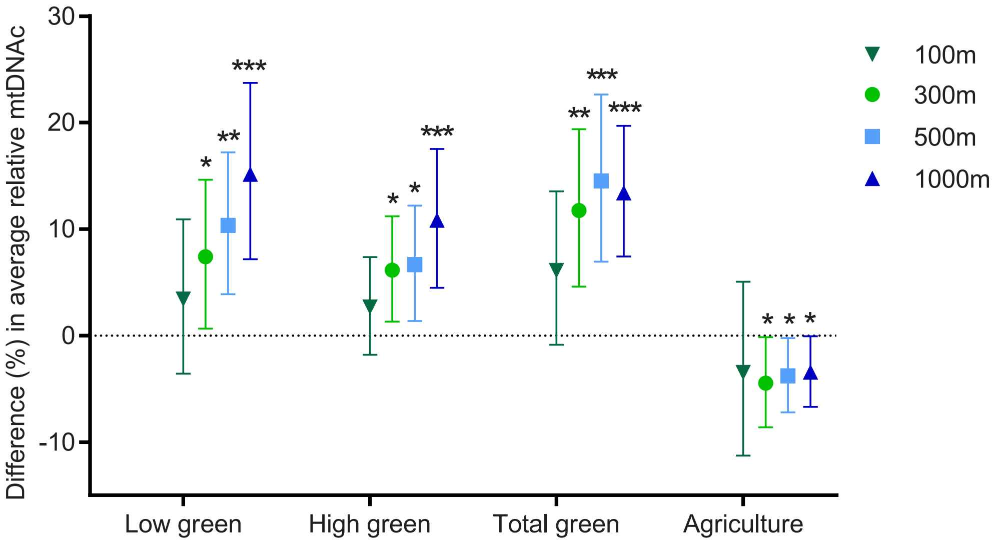

Fig. 1. Difference (%) in average relative mitochondrial DNA content (mtDNAc) (with 95% CI) in association with an IQR increment in residential green space (high green, low green, total green, and agriculture). Because of the skewedness of the data for the 100 m radius of agriculture, we expressed the effect size for a dichotomized variable comparing the presence to the absence of agriculture within this radius. Estimates were adjusted for sex, age, BMI, highest educational level of either parent, passive smoking, season, date of examination, distance to major road, and the random effect of school and subject. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Residential exposure characteristics (n = 246).

	Mean	Median	IQR
Low green (%)			
100 m radius	34.2	35.0	18.5
300 m radius	31.6	32.4	12.8
500 m radius	30.4	31.2	11.3
1000 m radius	28.7	31.5	11.0
High green (%)			
100 m radius	16.1	13.7	11.7
300 m radius	19.1	17.5	8.9
500 m radius	20.2	19.2	9.0
1000 m radius	20.9	20.2	9.5
Total green (%)			
100 m radius	50.2	52.7	22.0
300 m radius	50.7	52.7	18.6
500 m radius	50.6	52.9	18.8
1000 m radius	49.6	53.5	13.9
Agriculture (%)			
100 m radius	6.1	0.0	8.3
300 m radius	11.5	6.6	15.0
500 m radius	13.7	9.1	12.7
1000 m radius	17.0	13.5	11.6
Distance to green space >0.5 ha (m)	210.8	123.7	144.6

IQR: interquartile range; Low green: <3m; High green: ≥3m; Total green: sum of low and high green

agricultural area where an IQR increment (11.9%) in agricultural area in a 1000 m radius was associated with a −3.4% (95% CI: -6.7% to −0.1%, $p = 0.047$) lower mtDNAc (Fig. 1).

In addition, mtDNAc was inversely correlated with distance to green space (area with more than 0.5 ha of green). After adjustment, a doubling in distance was significantly associated with a −5.2% (95% CI: -7.9 to −2.4%, $p = 0.00040$) lower mtDNAc (Fig. 2).

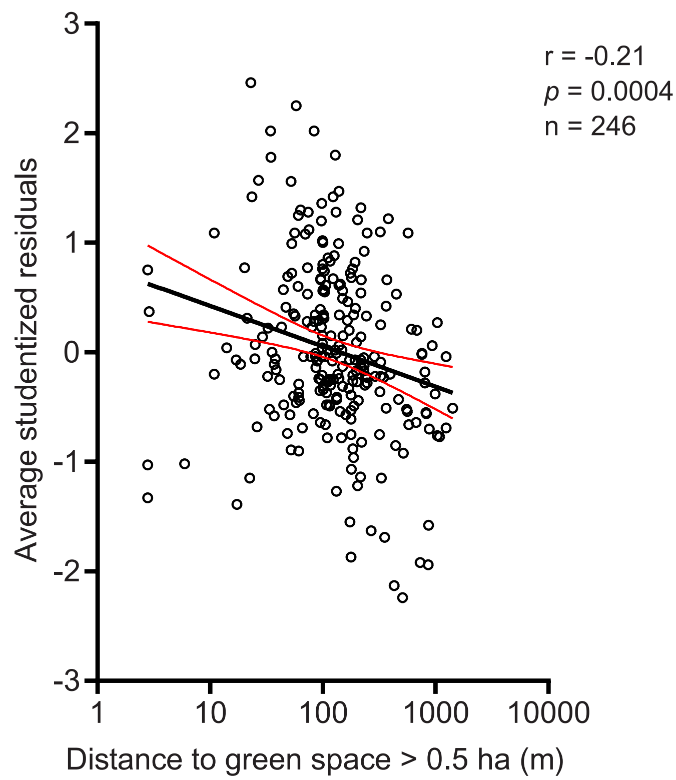

Fig. 2. Scatterplot showing the association between distance to green space > 0.5 ha (area with more than 0.5 ha of green) and average residualized mtDNAc per child. MtDNAc was residualized by regressing on the covariates (age, sex, BMI, passive smoking, season of examination, highest educational level of either parent, date of examination and distance to major road). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Sensitivity analysis

Average (SD) $PM_{2.5}$ exposure during the year before examination was 14.7 (1.1) μg/m^3. Average UFP (SD) exposure measured at school on the day of the examination was 23,604 (15,272.2) particles/cm^3. Additional adjustment of the main model for $PM_{2.5}$ exposure, UFP exposure and physical activity, separately or combined are presented in Table 3 for the 1000 m radius and in and supplementary table 3-5 for the other radii. Results did not show a notable change in the effect estimates of the aforementioned associations. However, after adjusting for physical activity, the results for agriculture showed slightly higher *p*-values. Also, only including the first measurement of each child did not alter the results (Suppl. table 6).

4. Discussion

In 9–12 year old children, we investigated whether residential surrounding green space and distance to green space were associated with mitochondrial DNA content. We found that an increase in surrounding

Table 3
Sensitivity analysis for the 1000 m radius and distance to green space.

	n	Mitochondrial DNA content (mtDNAc)		
		% Difference	95% CI	*p*-value
Low green	436	15.2	7.2 to 23.7	0.00014
+ Physical activity	424	14.4	6.1 to 23.3	0.00051
+ $PM_{2.5}$ exposure	436	14.2	6.3 to 22.8	0.00037
+ UFP exposure	436	15.2	7.2 to 23.8	0.00014
+ Physical activity, $PM_{2.5}$ and UFP	424	13.6	5.4 to 22.5	0.0010
High green	436	10.8	4.5 to 17.5	0.00070
+ Physical activity	424	10.2	3.7 to 17.1	0.0018
+ $PM_{2.5}$ exposure	436	9.9	3.4 to 16.7	0.0024
+ UFP exposure	436	10.7	4.4 to 17.4	0.00079
+ Physical activity, $PM_{2.5}$ and UFP	424	9.3	2.7 to 16.3	0.0051
Total green	436	13.4	7.4 to 19.7	<0.0001
+ Physical activity	424	13.0	6.7 to 19.6	<0.0001
+ $PM_{2.5}$ exposure	436	12.7	6.6 to 19.2	<0.0001
+ UFP exposure	436	13.3	7.4 to 19.7	<0.0001
+ Physical activity, $PM_{2.5}$ and UFP	424	12.4	6.0 to 19.1	0.00012
Agriculture	436	−3.4	−6.7 to −0.1	0.047
+ Physical activity	424	−3.0	−6.5 to 0.7	0.11
+ $PM_{2.5}$ exposure	436	−3.4	−6.7 to 0.1	0.055
+ UFP exposure	436	−3.4	−6.8 to 0.03	0.052
+ Physical activity, $PM_{2.5}$ and UFP	424	−2.9	−6.4 to 0.8	0.12
Distance to green space > 0.5 ha	436	−5.2	−7.9 to −2.4	0.00040
+ Physical activity	424	−4.9	−7.6 to −2.0	0.00098
+ $PM_{2.5}$	436	−5.0	−7.7 to −2.2	0.00057
+ UFP exposure	436	−5.2	−7.9 to −2.4	0.00036
+ Physical activity, $PM_{2.5}$ and UFP	424	−4.8	−7.5 to −1.9	0.0012

Percentage difference (95% CI) in relative average mitochondrial DNA content (mtDNAc) for an IQR increment in residential green space or a doubling in distance to green space >0.5 ha. The main model was adjusted for sex, age, BMI, highest educational level of either parent, passive smoking, season, date of examination, distance to major road, and the random effect of school and subject. In the sensitivity analysis, the models were additionally adjusted for physical activity, $PM_{2.5}$ exposure during the year before the examination, and ultrafine particle (UFP) < 300 nm exposure at school separately and combined.

green space was associated with a higher mtDNAc, whereas the opposite was found for agricultural area. Secondly, our results showed a higher mtDNAc for children living closer to green space.

4.1. Interpretation of the findings

To our knowledge, this is the first study to evaluate the association between green space exposure and mtDNAc. Although we cannot compare with other studies, our results are in line with several previous observations. More time spent in green spaces has been associated with lower stress, better mental health, and improved social contacts (Beyer et al., 2014; Brown et al., 2018; Dadvand et al., 2019), which in turn, could be associated with higher mtDNAc (Brown et al., 2018; Orban et al., 2017; Pun et al., 2018).

Furthermore, we found that green space within a 500 m and 1000 m radius had the strongest effect on mtDNAc. This is consistent with previous evidence where larger radii of green space were more strongly associated with health outcomes. For example, the 500 m radius compared with smaller radii was more strongly associated with birth weight and head circumference (Payam Dadvand et al., 2012b). Also, greenness within 3 km was associated with mental health whereas no association was found for 1 km (Bos et al., 2016). Finally, built greenness radii between 100 m and 2000 m showed stronger associations for larger radii and intelligence quotient in children (Lee et al., 2021).

However, two studies also found associations with health outcomes for smaller radii. A higher birthweight was found for exposure to green space in a 50 m and 100 m radius (Laurent et al., 2013) and the strongest association between green space and mental and self-perceived health was found for the 100 m and 250 m radius, respectively (Su et al., 2019). This indicates the possibility of different underlying mechanisms. Dadvand and colleagues hypothesized that visual access and heat reduction might be important mechanisms of immediate surrounding green space, whereas larger radii might be associated with other mechanisms like social health (Dadvand et al., 2016) and physical activity (Dadvand et al., 2014). Indeed, research showed that larger radii are more strongly associated with social support (Dadvand et al., 2016) and physical activity (Su et al., 2019), and that they better predict physical activity (Browning and Lee, 2017), which has been associated with higher mtDNAc (Steiner et al., 2011). Finally, not only surrounding green space but also access to a large green space is important in the context of physical activity (McCormack et al., 2010). The association of residential green space with mtDNAc is confirmed by our finding that living closer to green space was associated with a higher mtDNAc.

On the other hand, we found an inverse association between surrounding agricultural area and mtDNAc. One possible hypothesis could be that children living in an environment with more agricultural areas are exposed to a lower percentage of surrounding green space and therefore might experience less positive effects of green space. Our findings showed that with the green map of Flanders, green space was inversely associated with agriculture. Another explanation from literature could be that agricultural activities are associated with emissions of primary particulate matter (PM), nitrogen oxides (NO_x), and ammonia, which in turn give rise to the formation of secondary PM. These agricultural emissions have been associated with adverse health effects (Pozzer et al., 2017), and pesticides have been linked with oxidative stress and DNA damage (Kisby et al., 2009). However, additionally adjusting for long-term $PM_{2.5}$ in the sensitivity analysis did not alter the observed effect. Future studies should confirm the association between agriculture and mtDNAc and further elucidate underlying pathways.

4.2. Biological processes

The biological pathways underlying the association between green space and mtDNAc are not yet known. One hypothesis is oxidative stress and inflammation. Higher exposure to green space has been associated with higher levels of physical activity (Steiner et al., 2011), lower levels of obesity (Knobel et al., 2020), and lower levels of psychological stress (Beyer et al., 2014). All three factors have been associated with a decrease in oxidative stress and inflammation (Eick et al., 2018; McMurray et al., 2016; Simioni et al., 2018), which in turn has been associated with higher levels of mtDNAc (Castellani et al., 2020). Furthermore, as mtDNAc is strongly correlated with telomere length (Martens and Nawrot, 2016), our results support the research by Miri et al. (2020) which showed a positive effect between green space and telomere length in children (Miri et al., 2020).

Even though mtDNAc is often evaluated in adults and elderly, we believe that it is important to also assess these mtDNAc processes in early life because environmental exposures might already affect mtDNAc *in utero* as shown for multiple toxic metals by Vriens et al. (2017) and during childhood as shown for air pollution in our previous study (Hautekiet et al., 2020). The Developmental Origins of Health and Disease (DOHaD) hypothesis states that adverse influences of the early-life environment can result in permanent changes in adulthood (De Boo and Harding, 2006). Accordingly, the positive effect of green space in early life has been shown to be associated with health outcomes later in life such as blood pressure (Bijnens et al., 2017) and mental health (Engemann et al., 2019). Therefore, increasing exposure to beneficial environments, like green space, during early life might promote health in later life.

4.3. Strengths and limitations

The main feature of our study is that it is the first to explore the associations of surrounding green space with mtDNAc in children. Other strengths are the use of a repeated measures design and the fact that we were able to estimate surrounding green space using a specific high-resolution map. However, the latter makes it less evident to compare our results with other studies which often use the normalized difference vegetation index (NDVI, which is an indicator of greenness) or the Corine land cover (which is a coarse land cover map in which smaller green spaces are dissolved in mixed pixels). We chose to use the green map of Flanders in this study as it captures green space on a high-resolution (1m × 1m), whereas CORINE land-cover data does only include green areas that are at least 25 ha and NDVI often fails to distinguish between natural and agricultural areas.

A limitation of our study is the use of buccal swabs. Because children are the subjects of our study, we used buccal swabs to sample DNA as this is a common, non-invasive method compared to the drawing of blood. However, there are three disadvantages to the use of buccal swabs. Firstly, the quality of the obtained DNA is lower (Hansen et al., 2007), which resulted in a loss of data. Secondly, we were not able to compare the mtDNAc of buccal swabs with other somatic cells. Therefore, our results should be interpreted with caution. Finally, the oral cell composition might be influenced by poor oral hygiene or infection (Shalev, 2012). However, to avoid this, we used a standardized protocol in which the children rinsed their mouth three times with water and were not allowed to eat or drink 30 min before sampling. Lastly, the study design and statistical approach including the repeated measurements caused a lower risk of reverse causality. However, as for multiple children only one measurement was available, the power of the study design declines so caution is advised when interpreting the causality of these results.

5. Conclusion

In the present study, we found a positive association of buccal mtDNAc with residential surrounding green space and an inverse association with agricultural area. We also showed that living closer to large green space of >0.5 ha was positively associated with mtDNAc. To what extent higher mtDNAc due to green space contributes to human health effects should be further elucidated.

Author contributions

Pauline Hautekiet: Methodology, Formal analysis, Writing – original draft, Visualization; **Nelly D. Saenen**: Conceptualization, Investigation, Methodology, Writing – review & editing, Data curation, Project administration; **Raf Aerts**: Writing – review & editing; **Dries S. Martens**: Methodology, Writing – review & editing; **Esmée M. Bijnens**: Methodology, Writing – review & editing; **Harry A. Roels**: Writing – review & editing; **Tim S. Nawrot**: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Validation, Funding acquisition, Project administration.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.113551.

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