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Inge Bos^{1,2}, Patrick De Boever^{1,3}, Jef Vanparijs², Nathalie Pattyn^{2,4},
Luc Int Panis^{1,5}, and Romain Meeusen²

¹Environmental Risk and Health, Flemish Institute for Technological Research (VITO), Mol, Belgium; ²Human Physiology & Sports Medicine, Vrije Universiteit Brussel, Belgium; ³Centre for Environmental Sciences (CMK), Hasselt University, Diepenbeek, Belgium; ⁴VIPER Research Unit, Royal Military Academy, Brussels, Belgium; ⁵Transportation Research Institute (IMOB), Hasselt University, Diepenbeek, Belgium

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Inge Bos^{1,2}, Patrick De Boever^{1,3}, Jef Vanparijs², Nathalie Pattyn^{2,4}, Luc Int Panis^{1,5}, and Romain Meeusen²

¹ Environmental Risk and Health, Flemish Institute for Technological Research (VITO), Mol, Belgium;

² Human Physiology & Sports Medicine, Vrije Universiteit Brussel, Belgium;

³ Centre for Environmental Sciences (CMK), Hasselt University, Diepenbeek, Belgium;

⁴ VIPER Research Unit, Royal Military Academy, Brussels, Belgium;

⁵ Transportation Research Institute (IMOB), Hasselt University, Diepenbeek, Belgium

* Corresponding author: R. Meeusen at Faculty of Physical Education and Physiotherapy, Department of Human Physiology and Sports Medicine, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium. Tel.: +32 2 6292222; Fax: +32 2 6292876. E-mail address: rmeeusen@vub.ac.be

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Abstract

Purpose: Particulate Matter (PM) exposure is linked to inflammation, neuroinflammation and cognitive decline, whereas aerobic training improves cognition.

We investigated the effects of PM exposure during aerobic training on inflammatory biomarkers, serum Brain-Derived Neurotrophic Factor (BDNF), an assumed mediator of exercise-induced cognitive improvements, and cognitive performance.

Methods: Two groups of untrained volunteers completed an aerobic training program of 12 weeks, 3 sessions/week, one group (n = 15) in an urban, another group (n = 9) in a rural environment. **Ultrafine particulate matter (UFPM) concentrations were measured during each training session.** Aerobic fitness (Cooper test), BDNF serum levels, **blood total and differential leukocyte counts**, exhaled Nitric Oxide (eNO) levels and cognitive performance (Stroop task, Operation Span, Psychomotor Vigilance Task) were analyzed before and after the program.

Results: UFPM concentrations were significantly higher in the urban environment compared to the rural environment ($p = 0.006$). Fitness levels improved equally ($p < 0.0001$) in both groups. Leukocyte counts ($p = 0.02$), neutrophil counts ($p = 0.04$), and eNO levels ($p = 0.002$) increased after training in the urban group, whereas these parameters did not change in the rural group. **The changes in these markers' levels after training showed a positive correlation with the personal, average UFPM exposure during training.** Reaction times on the Stroop task improved in the rural group ($p = 0.001$), but not in the urban group. No effects were found on BDNF levels, Operation Span and Psychomotor Vigilance test performances.

Conclusion: Aerobic training in an urban environment with high traffic-related air pollution increased inflammatory biomarkers and, in contrast to aerobic training in a rural environment, cognitive performance on the Stroop task did not improve.

Keywords: air pollution, particulate matter, exercise, inflammation, cognition, Brain-Derived Neurotrophic Factor (BDNF).

Introduction

Paragraph number 1 Urbanization is an upcoming process. Today, more than half of the world's population is living in urban areas (36). However, the dense traffic network that crosses cities and urbanized environments, provokes high levels of traffic-related air pollution. **Traffic exhaust generated by combustion processes in the engines of the vehicles is an important source of particulate matter (PM) and, in particular, ultrafine particulate matter (UFPM) that is characterized by an aerodynamic diameter equal to or smaller than 100 nm (26).**

Paragraph number 2 Exposure to air pollution is associated with negative health effects and respiratory and cardiovascular effects are well documented (7). It is hypothesized that inflammation is an important part of the mechanism through which PM induces negative health effects, as markers of respiratory and systemic inflammation are increased in response to PM exposure (6, 7, 25, 37). More recently, associations were found between chronic exposure to air pollution and negative neurological effects. Calderon and colleagues detected associations between residence in a highly polluted city rich in ozone and PM, and ultrafine particle deposition in the brain, neuroinflammation, disruption of blood-brain barrier,

accumulation of amyloid- β and α -synuclein, and cognitive decline (9, 10, 11). In addition to Calderon and colleagues, others have found associations between PM exposure and cognitive decline (30).

Paragraph number 3 Physical activity is known to improve health as demonstrated by the cardiovascular benefits. Positive effects are also documented for brain plasticity, cognition and mental health (3, 13, 23, 28, 29, 35, 38, 39). Participation in an aerobic training program, for example, was previously found to improve cognitive domains such as cognitive flexibility and visuospatial memory in healthy subjects (29, 35). The exact mechanism that underlies these functional benefits of exercise remains to be elucidated. It was demonstrated in rodents that exercise stimulates brain plasticity processes including neurogenesis and synaptic plasticity and increases brain levels of neurotrophic growth factors that stimulate neuron differentiation, growth and survival (28, 38, 39). Moreover, it was shown that brain-derived neurotrophic factor (BDNF, a neurotrophine) plays a major role in the improved learning and memory in response to exercise since blocking the action of BDNF inhibits the improvements (39). Also in humans, the peripheral levels of BDNF are transiently increased during and after an acute bout of exercise and it is suggested that the peripheral increase is reflected in the brain where it may contribute to the above mentioned functional benefits (19, 22, 31). We recently found evidence suggesting that the exercise-induced increase in BDNF serum level is suppressed by PM exposure while cycling near a busy traffic road. Healthy participants performed two 20 minute cycling tests of the same intensity, once in a room where PM₁₀, PM_{2.5} and **UFPM** was filtered out of the air and once on a cycling path along a busy road where concentrations of PM₁₀, PM_{2.5} and **UFPM** were significantly higher. Consistent with literature, serum BDNF level increased after the cycling test in the air-filtered room, but in contrast no increases in BDNF levels were found after the cycling test along the busy road (5). Additionally, in response to cycling along the busy road an increase was found in the

fraction of blood neutrophils, a marker of inflammation (25). In the present study, we investigate the effect of PM exposure during aerobic training on markers of systemic and respiratory inflammation, more specifically differential leukocyte counts and exhaled Nitric Oxide (eNO), cognition and basal BDNF levels. We hypothesize that chronic PM exposure during aerobic training may increase markers of inflammation, decrease basal BDNF levels and may affect cognitive performance.

Methods

Paragraph number 4 Participants. Thirty-four untrained, healthy participants were recruited for this study. Twenty-one participants were recruited at the Vrije Universiteit Brussel (VUB), located in Brussels (Belgium), and 13 participants were recruited at the Flemish Institute for Technological research (VITO), located in Mol (Belgium). The intake procedure of the subjects consisted of a medical examination, including systemic, familial and sports anamnesis, medication, general clinical examination, skinfold measurement, basic spirometry, rest electrocardiogram (ECG), ECG during a maximal effort test until exhaustion and during recovery. The maximal effort test with ECG and spirometry measurements was included in the intake to make sure that there were no contraindications for sports participation and to examine basic VO_2 max. In addition, a questionnaire was administered to collect extra information on lifestyle activities, physical activity level, educational background and personal exposure to air pollution. Inclusion criteria were (1) untrained subjects (no aerobic training for at least 3 months), (2) age between 18 - 60 years, (3) BMI between 20 - 30. Exclusion criteria were (1) contraindications for sports participation, (2) lung diseases (such as asthma, hay fever), (3) neurologic diseases. The study was approved by the Ethics Review Board of the Medical Faculty of the VUB. All subjects gave a written informed consent.

Paragraph number 5 Study design. Two groups of subjects participated in an aerobic training program from February, 9th, 2011 until May, 2nd, 2011. One group trained in an environment with high traffic-related air pollution (Brussels, urban group), the other group in an environment with lower traffic-related air pollution (Mol, rural group). The training sessions were organized on the athletics track of the VUB-campus Etterbeek in the city of Brussels (urban group), and at the Nuclea athletics track in the rural environment of Mol (rural group). We used a “Start to run” program, an aerobic training program designed for formerly inactive people (**Supplementary table S1**, Total duration of the training sessions (minutes) with the respective dates and the duration of walking and running specified). The “Start to run” program was given in organized sessions for 12 weeks, with weekly work-out sessions on Monday, Wednesday and Friday between 12 A.M. and 1 P.M.. In this aerobic training program walking and running are alternated and the duration of the training and running intervals increase every 3 training sessions. At the first training session the total duration was 20 minutes - including 10 minutes of running - and at the final session the total duration was 32 minutes of running only (**Supplementary table S1**, Total duration of the training sessions (minutes) with the respective dates and the duration of walking and running specified). Participants received heart frequency monitors and guidelines to train aerobically around 75% of their maximal heart rate. Training participation was monitored in each group. Aerobic fitness, differential leukocyte counts, exhaled NO (eNO) levels, serum BDNF levels and cognitive performances on the Stroop Color Word test, Automated Operation Span test and Psychomotor Vigilance test were measured before and after the training program.

Paragraph number 6 Exposure measurements. During every work-out, the average particle number concentration of particles in the size range 0.02 – 1 µm was measured on the athletics tracks using TSI P-TRAK UFP Counters (TSI Model 8525, USA). **The location of the counters on the track was chosen by performing preliminary measurements to**

determine where the UFPM concentration on the track represents approximately the average of the minimal and the maximal UFPM concentration measured on the track (data not shown). The particle counters were located along the right-sided straight line of the athletics track at 50 – 100 cm above the ground. During periods of rain, the counters were placed under the nearest roof on the athletics track.

Outcome measures

Paragraph number 7 Aerobic fitness level. Improvement of aerobic fitness at the end of the program was evaluated by measuring the individual performances on the Cooper test before and after the program. During this field test, participants had to walk and/or run at a steady pace for as much distance as possible in 12 minutes.

Paragraph number 8 Exhaled NO. Fractional exhaled NO was measured with an electrochemistry-based NIOX MINO device (Aerocrine, Sweden). The procedure consists of a maximal inhalation through the device that contains a scrubber which makes the inhaled air NO-free. Inhalation is followed by expiration guided by the device to maintain a flow rate of 50ml/second.

Paragraph number 9 Blood collection and analyses. Non-fasting venous blood samples were collected in an EDTA tube (BD Vacutainer®, USA) and in a serum-separator tube (BD Vacutainer®, USA). Blood leukocyte counts and differential leukocyte counts were determined using an automated cell counter Sysmex SF3000 Hematology Analyzer (Sysmex Corporation, Kobe, Japan). BDNF concentrations in serum were analyzed by enzyme immunoassay using ELISA kits by Chemicon (Temecula, CA, USA).

Cognitive testing

Paragraph number 10 The Stroop Color Word Test. The Stroop Color Word Task measures response-inhibition and selective attention which are functional parts of the executive function or higher level cognition (2, 33). During the automated Stroop Color Word test, a word or “xxxx” written in red, green, blue or yellow is presented in the middle of the screen, in bold Courier New font, 14 points, under a vertical visual angle of 2° 03’. The subjects have to respond to the color of the word by pressing the corresponding color-button in the keyboard as fast as possible. The complexness of the test is made by the inclusion of multiple conditions, i.e. the congruent condition (a word describes its own color, e.g., the word “blue” printed in blue), the incongruent condition (a word describes a different color from its own, e.g., the word “blue” printed in green), the no-word condition (e.g., “xxxx” printed in blue), the simple negative priming condition (the to-be-ignored stimulus becomes the subsequent relevant response, e.g., the word *red* displayed in green immediately followed by the word *blue* displayed in red), the inverse negative priming condition (the relevant response becomes the to-be-ignored stimulus, e.g., the word *red* displayed in green immediately followed by the word *green* displayed in red). The response-stimulus interval (RSI) was 32 ms. The tasks began with detailed instruction screen, followed by a 60 trials practice block during which the subjects received feedback regarding their performance (“correct” or “incorrect” and response time), before the experimental block. Response times and accuracy were recorded. The task was programmed and run using E-prime® (Copyright © 2002 Psychology Software Tools, Pittsburgh, USA).

Paragraph number 11 The OSPAN test. The operation span (OSPAN) test assesses working memory, which is part of the executive functions (2, 14). The OSPAN task with mathematical processing is based on Conway & Engle, 1996 (14). In this task, the subjects are presented with a series of simple math operations, to which they have to respond ‘False’ or ‘True’,

alternated with a letter-to-be recalled presented on the screen for 800ms. A series consists of 3 to 7 math operations alternated with a letter to-be-recalled. At the end of a series, they have to recall the letters in the exact order as they appeared by clicking the box next to the appropriate letters. The task begins with an instruction list and 3 practice blocks to familiarize the subjects with each task. The first practice block is on letter recall, followed by a practice block on math operations, and a final practice block in which they have to perform both math operations and letter recall together, just as they will do in the experimental block. During the practice block on math operations the individual's mean time required to solve the math problems is calculated. This time (plus 2.5 standard deviations) is then used as a time limit for the math portion of the experimental session, to prevent the subject from rehearsing the letters during the math operation. The experimental block consist of 3 sets of each set-size (ranging from 3 - 7 math operations). The subjects are instructed to keep their math accuracy, which is displayed in the upper-right corner of the screen during recall, at or above 85% at all times. The program reports 5 values: the OSPAN score or the sum of all perfectly recalled series, e.g. if an individual recalled correctly 3 letters in a set size of 3, and 4 letters in a set size of 4, and 4 letters in a set size of 5, the OSPAN score is 7 (3 + 4 + 0), the Total Number Correct or the total number of letters recalled in the correct position, and math errors reported as Total Number of Errors, Accuracy Errors where the subject solved the operation incorrectly, and Speed Errors in which the subject ran out of time in attempting to solve a given operation.

Paragraph number 12 The PVT. The PVT measures vigilant or sustained attention and reaction time (16). The task is based on a simple visual reaction time test apparatus originally developed by Wilkinson and Houghton, 1982 (40). The PVT ran for a duration of 10 min. The subjects were asked to respond to a visual stimulus presented at a variable interval (2 – 10 sec) by pressing either the right or the left mouse button with the index finger of the dominant hand. The visual stimulus was the three-digit counter turning on and incrementing from 0 to

500 ms at 1-msec intervals. Upon pressing the button, the counter display stopped incrementing, allowing the subject 1 sec to read his or her reaction time before the counter restarted. A trial in which a response was not made within 500 ms was stored as a lapse, warning the subjects through a message on the screen. The task began with an instruction screen and a practice block followed by the experimental block. Reaction time of correct responses (i.e. RT under 500 ms) and number of lapses (i.e. the number of responses where the RT exceeded 500 ms) were stored.

Statistical analyses

Paragraph number 13 Data normality, homogeneity of variance and the assumption of sphericity were assessed whenever necessary through the Kolmogorov-Smirnov test, the Levene's test and Mauchli's test of sphericity respectively. Parametric tests were used in case of normally distributed data and equal variances, in other cases nonparametric tests were used. The significance value was set at $P \leq 0.05$. The statistical analyses were performed using IBM SPSS Statistics version 20 for Windows software.

Paragraph number 14 Parametric tests. Reaction time in the Stroop Color Word task was analyzed using a 5 (condition) x 2 (time) x 2 (location) ANOVA with condition (congruent - no-word - incongruent - simple negative priming – inverse negative priming) and time (pre – post) as within-subjects factors and location (Brussels – Mol) as a between-subjects factor. In case of a significant F value, separate ANOVA's were performed, including 5 (condition) x 2 (location) ANOVA's to analyze group differences at PRE and POST intervention time and 5 (condition) x 2 (time) ANOVA's to analyze the effect of training in each group. A 2 (time) x 2 (location) ANOVA with time as a within-subjects factor and location as a between-subjects factor was used to analyze the effect of aerobic training (time) and training location (location)

on blood leukocyte counts and differential leukocyte counts (counts of neutrophils, lymphocytes and eosinophils), and OSPAN score and Total Number of Errors in the OSPAN task. Whenever a significant F value was obtained in ANOVA, the differences between the groups were analyzed using independent samples T-tests to identify group differences PRE and POST intervention and paired samples T-tests were performed to analyze the effect of training in each group. Baseline characteristics (Weight, height, BMI) were compared between the groups using an independent samples T- test. The effect of training on Cooper test performances and BDNF levels was analyzed using a paired samples T-test.

Paragraph number 15 Nonparametric tests. A Mann Whitney U Test was used to compare UFPM concentrations between the training locations, to compare baseline characteristics (age, HR_{max}, Watt_{max}, VO_{2max}) and to analyze group differences in the Cooper test performance, eNO levels, BDNF levels, blood counts of monocytes and basophils, performance in the PVT (accuracy and reaction time), Stroop test (accuracy), and OSPAN task (Total Number of Errors, Accuracy Errors, Speed Errors) PRE intervention and POST intervention. The effect of aerobic training on eNO levels, blood differential counts (counts of monocytes and basophils), performances in the PVT (accuracy and reaction time), Stroop task (accuracy), and OSPAN task (Total Number of Errors, Accuracy Errors, Speed Errors) was analyzed for each group using the Wilcoxon Signed-Rank Test. **The correlation between the personal, average UFPM exposure during training and the change in concentration of total and differential leukocytes, and eNO was analyzed using the Spearman's correlation coefficient. We calculated the personal average UFPM exposure during training on the basis that every subject has followed a unique set of training sessions. The average UFPM concentration measured during a specific training was multiplied by the duration (minutes) of that session and per subject the sum of all training session products in which they participated was calculated. The amount of time spent in**

training was different for each subject, we therefore corrected the personal cumulative UFPM exposure during training for total amount of time spent in training (minutes). In other words, we calculated an approximation of the average UFPM the participants were exposed to per minute training and correlated that value to the changes in total and differential leukocyte counts and the eNO levels after training.

Results

Study population

Paragraph number 16 The Start to run was initiated with 21 and 13 individuals in the urban and rural group, respectively. During the exercise program, 4 participants of the rural group and 4 participants of the urban group suffered from various injuries preventing them from participating to the remaining training sessions. Participants who did not take part in at least 16 training sessions were excluded from the analyses and for that reason 2 more participants of the urban group were excluded. Finally, 15 participants from the urban group and 9 participants from the rural group completed the training program. Characteristics of the two study populations are summarized in Table 1. The groups were comparable, except for the average age, which was significantly higher in the rural group.

Exposure measurements

Paragraph number 17 The average particle number measured per training session on the athletics track at the urban and the rural location is shown in figure 1. The average particle number measured at each location differed significantly (Mann Whitney U test,

U= 393), p=0.006, with 7244 (2559) particles/cc at the urban and 5625 (1896) particles/cc at the rural location.

Outcome measures

Paragraph number 18 Aerobic fitness. Both groups improved their performance on the Cooper test, $t(13) = -6.93$, $p < 0.001$, $r = 0.89$ and $t(7) = -6.42$, $p < 0.001$, $r = 0.92$ for the urban and rural group respectively (table 2). The mean performance improvements, as calculated by the difference in performance POST minus PRE training, did not differ between the groups, $t(20) = -1.46$, $p = 0.16$. The Cooper test performance pre training and post training did not differ between the groups, $U = 50.5$, $p = 0.73$, and $U = 44$, $p = 0.43$ respectively.

Paragraph number 19 Exhaled NO. The eNO levels increased significantly, $Z = -2.87$, $p = 0.002$, $r = -0.76$, in the urban group, whereas eNO levels did not change, $Z = -0.7$, $p = 0.52$, $r = -0.25$ in the rural group (table 2). There was no significant difference in eNO levels before the training, $U = 56.5$, $p = 0.7$. **Personal, average UFPM exposure during training positively correlated with the percentage change of the eNO levels, $r_s = 0.51$, $p = 0.03$ (Fig 2A), but not significantly with the absolute change in eNO levels or $\Delta(eNO)_{\text{post-pre}}$, $r_s = 0.40$, $p = 0.099$.**

Paragraph number 20 Total leukocyte count and differential count. The ANOVA revealed no significant main effect of time, $F(1,21) = 0.51$, $p = 0.49$, nor location, $F(1,21) = 0.042$, $p = 0.84$, although there was a significant interaction between time and location for the total leukocyte count, $F(1,21) = 4.49$, $p = 0.046$. Post hoc comparison showed that there was no difference, $t(21) = -1.64$, $p = 0.12$, in total leukocyte count between both training groups before the training intervention. Total leukocyte count in the urban group increased significantly, $t(13) =$

- 2.61, $p = 0.02$, $r = 0.59$, in response to the training program, while total leukocyte count did not differ significantly over time in the rural group, $t(8) = 0.76$, $p = 0.47$, $r = 0.26$ (table 2). The increase in total leukocyte count in Brussels was the consequence of an increase in neutrophil count, $t(13) = - 2.25$, $p = 0.04$, and a trend towards an increase in lymphocyte count, $t(13) = - 1.87$, $p = 0.08$ (table 2). No effects were found on counts of eosinophils, monocytes and basophils (table 2). **Personal, average UFPM exposure during training positively correlated with the absolute change in white blood cell (WBC) counts or $\Delta(\text{WBC counts})_{\text{post-pre}}$, $r_s = 0.54$, $p = 0.02$ and the percentage change of WBC counts, $r_s = 0.53$, $p = 0.02$ (Fig 2B) as well as the absolute change in neutrophil counts or $\Delta(\text{neutrophil counts})_{\text{post-pre}}$, $r_s = 0.46$, $p = 0.049$. There was a trend towards a positive correlation with the percentage change of the neutrophil counts, $r_s = 0.44$, $p = 0.058$ (Fig 2C), as well as the percentage, $r_s = 0.43$, $p = 0.066$ (Fig 2D), and the absolute change, $r_s = 0.44$, $p = 0.057$ of the lymphocyte counts. There were no correlations between the personal UFPM exposure during training and variations in the counts of the eosinophils, the monocytes and the basophils.**

Paragraph number 21 BDNF concentrations in serum. There were no group differences in BDNF levels before, $U = 54$, $p = 0.45$, and after, $U = 60$, $p = 0.68$, the intervention. There were no significant effects on BDNF concentrations in any of the groups, although there was a trend towards a decreased BDNF level, $t(8) = 1.97$, $p = 0.08$, in the rural group (table 2).

Paragraph number 22 Cognitive testing. The $5 \times 2 \times 2$ ANOVA revealed that there was a significant main effect of condition, $F(2.44, 88) = 5.52$; $p = 0.004$, and time, $F(1, 22) = 8.7$; $p = 0.007$, and a significant interaction between time and location, $F(1, 22) = 4.66$; $p = 0.04$, on reaction time in the Stroop task. There was no significant main effect of location,

$F(1,22)=0.47$, $p=0.50$, and no significant interaction between condition and location, $F(4,88)=0.041$; $p=1.00$, between condition and time, $F(4,88)=0.79$; $p=0.54$, and between condition, location and time, $F(4,88)=0.31$; $p=0.87$. The 5 x 2 (location) ANOVA on pre-tests showed no main effect of location, $F(1,22)=1.7$, $p=0.21$. The 5 x 2 ANOVA for the rural group showed a significant effect of condition, $F(4,32)=2.94$, $p=0.04$, and time $F(1,8)=24.4$, $p=0.001$. There was no significant interaction between time and condition $F(4,32)=0.54$, $p=0.70$. Post hoc analysis showed that reaction times of the rural group significantly improved in the no-word condition, from 841 (73) ms to 777 (61) ms, $t(8)=2.59$, $p=0.03$, $r=0.68$, in the incongruent condition, from 898 (93) ms to 820 (75) ms, $t(8)=4.50$, $p=0.002$, $r=0.85$, in the simple negative priming condition, from 880 (102) ms to 809 (63) ms, $t(8)=3.17$, $p=0.01$, $r=0.75$, and in the inverse negative priming condition, from 861 (89) ms to 810 (88) ms, $t(8)=3.70$, $p=0.006$, $r=0.79$ (**Fig 3**). The 5 x 2 ANOVA for the urban group showed a significant main effect of condition, $F(2.24,56)=3.34$, $p=0.04$, but no significant effect of time, $F(1,14)=0.31$, $p=0.58$ (**Fig 3**), and no significant interaction between condition and time, $F(4,56)=0.66$, $p=0.63$. There were no significant effects on accuracy in the Stroop task, and performance in the PVT and OSPAN task.

Discussion

Paragraph number 23 In this study, we find improved cognitive performance on a Stroop task in healthy subjects in response to aerobic training in a rural environment but not in response to aerobic training in an urban environment where traffic-related air pollution was substantially higher. In addition, the levels of inflammatory markers, more specifically blood leukocyte counts, neutrophil counts and eNO levels, were increased in the subjects who trained in the urban environment. **The change in the total leukocyte counts, neutrophil**

counts and the eNO levels after training showed a positive correlation with the personal UFPM exposure during training. The basal level of BDNF in serum was not significantly affected by exercise training in neither of the locations, although a trend towards a decreased BDNF level was found in the rural group.

Paragraph number 24 In this study, healthy, sedentary subjects improved their performance in the Stroop Color Word task, witnessed by the reduced reaction times, in response to two months of aerobic training in a rural environment. This is in agreement with previous intervention studies that also found improved performance on cognitive tasks in healthy subjects after participation in an aerobic training program (29, 35). The Stroop Color Word Task measures response-inhibition and selective attention, two processes that are component parts of the executive functions or “higher-level” cognitive functions involved in the control and regulation of “lower-level” cognitive processes and goal-directed future-oriented behavior as stated in Alvarez and Emory (2, 33). The neuroanatomical sites that are associated with Stroop task performance are the frontal lobes predominantly, the hippocampus and a number of neocortical regions (33). Our finding that simple reaction time and vigilance, measured by the Psychomotor Vigilance Test, did not improve with aerobic training, demonstrates that the improvements in the Stroop task did not occur simply as a result of improved reaction time or vigilance but rather as an improvement of the higher-level cognitive functions. In support with our findings, Masley et al (29) previously showed improvements in executive functions, more specifically on the shifting attention task and also on the Stroop task. Moreover, in a meta-analysis study, Colcombe and Kramer (13) concluded that the largest benefits of fitness occurred on the executive functions. However, in our study, the performances in the OSPAN test - a measure of working memory that is also a component part of the executive functions - did not improve with aerobic training (2, 14).

Paragraph number 25 In contrast, the group that exercised in the urban environment did not show an improvement on the Stroop Color Word Task, although the training protocol was identical and the number of training sessions followed by the participants did not differ between the groups. The improvements in fitness level did not differ from the rural training group. We suggest that these contradicting findings may be the consequence of a significant contrast in traffic-related air pollution between the two training locations. The concentration of **UFPM**, a marker of local traffic-related air pollution that was measured during the training sessions, was significantly higher in the urban training location compared to the rural location. **Long term PM exposure has been linked previously to neuroinflammation and cognitive decline in humans as well as animals (9, 10, 11, 20, 30).**

Paragraph number 26 The lack of cognitive improvements in the training group exposed to higher levels of air pollution supports our hypothesis based on findings in humans (5). Previously, we found evidence suggesting that PM exposure during a single exercise bout may inhibit the exercise-induced transient increase in serum BDNF levels. More specifically, an increased BDNF level was found after a cycling test in an air-filtered room but not after a cycling test equal in duration and intensity performed along a busy traffic road with substantially higher particle concentrations (5). It is suggested that the transient increase in the serum BDNF level in response to a single exercise bout is reflected by an increased BDNF level in the brain, which in its turn may be responsible for the benefits of exercise on cognition (19, 22, 31, 39). The association of the BDNF VAL66MET polymorphism with performance on an executive function test indeed suggests a role for BDNF in the executive function (27). From our previous findings, we hypothesized that air pollution exposure during aerobic training may interfere with the exercise-induced cognitive improvements, which is supported by the findings of this study.

Paragraph number 27 In this study, the subjects that exercised in the urban environment showed increased levels of inflammatory markers in response to aerobic training, whereas the rural training group did not. More specifically, blood leukocyte counts and neutrophil counts, markers of systemic inflammation, as well as the levels of eNO, a marker of respiratory inflammation, were increased in the urban training group. **In addition, the absolute and the percentage change of these markers correlated positively with the personal average UFPM exposure during training.** These inflammatory markers were previously associated with PM exposure (1, 7, 25, 37). **Moreover, inflammation is considered as one of the main mechanisms through which PM exposure induces negative health effects, also in the brain (6, 7).** This suggests that the exposure to air pollution during the training sessions in the urban area elicited an effect on systemic and respiratory inflammatory markers. Although the duration of the training sessions constitutes only a very small part of the total exposure time, it may still have a strong impact on total exposure because the increased ventilation during exercise significantly increases exposure (17, 24). **It is suggested that PM exposure may cause neuroinflammation indirectly by circulating inflammatory markers that reach the brain (6).** There is evidence linking peripheral inflammatory events to cognitive decline. **In older individuals, for example, an inflammatory event like injury, surgery or infection is often accompanied by a decline in cognition (18, 34).** Studies in aged rodents show that a peripheral inflammatory event, may lead to increased levels of inflammatory markers in the brain that in turn seem to be responsible for the cognitive decline through an inflammation-evoked reduction of BDNF signaling (4, 15).

Paragraph number 28 We did not find a significant effect of aerobic training on the basal level of BDNF in serum, although we noted a trend towards a decreased BDNF level in the rural group. While there is consistent evidence that a single bout of exercise transiently increases BDNF levels (19, 22), the effect of aerobic training on basal BDNF levels is less

clear. Some studies find reduced BDNF levels in trained subjects compared to inactive subjects (12). However, most studies report no effect of aerobic training on basal BDNF levels, which is in support with our findings (21, 32). **However, in the absence of more extensive air pollutant data it is difficult to interpret the current results.**

Paragraph number 29 Other indicators of urban air pollution, especially traffic-related compounds such as Black Carbon or nanometer sized particles, could have contributed to the contrast between the urban and the rural environment. For example, during one training session we included additional **UFPM** measurements of particles smaller than 20 nm and these measurements showed a peak (2.4×10^4 particles/cc) of 10 nm sized particles in the urban location whereas this peak was not present in the rural training group (data not shown). The duration of the exposure during the training sessions constitutes only a small part of the total exposure time. For logistical reasons we were not able to collect 24 hrs data on personal air pollution exposure. The possibility that an increased air pollution exposure at the time of the second sampling period was responsible for the effects measured in the urban group was also investigated. To this end, air quality data from the automatic monitoring network confirmed that the air pollution (PM₁₀, PM_{2.5}, Black Carbon, NO₂) in both locations during the second sampling period was not higher, but rather lower than during the first sampling period (data not shown). **We recognize that the lack of a cognitive positive response to training in the urban group may relate not necessarily to the air pollution exposure during the training period, but may be an overall response reflecting the personal chronic exposure to higher levels of air pollutants that in turn induce neuroinflammation.**

Paragraph number 30 We recognize some limitations of this study. First, there was a small but significant difference in age between both training groups. **The study shows improved cognitive performance in the rural group in response to training in spite they are older than the urban group.** The executive functions were previously shown to decline with age

(2, 8, 33). Therefore, due to the older age the rural training group may be more sensitive to the exercise-induced cognitive benefits. However, in this study, we observed no difference in cognitive performances at baseline between the groups, suggesting that the age difference was too small to affect cognition. **Alternatively, taking into account the age difference, we might speculate that the urban group may already be at a lower cognitive baseline performance as a result of a higher chronic exposure to air pollutants at baseline.** Secondly, an inactive control group was not included. We recognize that the inclusion of such a group would give more weight to the evidence and may reduce the risk of confounding effects. **Future experiments should include measurements of the inflammatory markers and BDNF levels before and after a training session as it may improve our understanding of the chronic effects.**

Paragraph number 31 **These new findings might be valuable for medical health workers and others that advise people to exercise outdoors in urban polluted environments. It can be discussed that the negative effects of systemic inflammation, cardiovascular effects, and neuroinflammation, etc. do not justify the “fitness” of the exposed outdoors individual.**

Conclusions

Paragraph number 32 **Our findings suggest that exercising regularly in an urban environment with high traffic-related air pollution exposure increases markers of respiratory and systemic inflammation. In addition, this study provides evidence suggesting that PM exposure during aerobic training inhibits the exercise-induced cognitive improvements.**

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Conflict of interest

None declared.

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Figure captions

Fig 1: Average particle number (particles/cc) measured per training session date on the athletics track at the urban and the rural training locations

Fig 2: Scatter plot with linear trend line, equation and coefficient of determination (R^2) of the relationship between the personal, average UFPM exposure during training with the percentage of the change in the exhaled Nitric Oxide (eNO) levels (A), white blood cell (WBC) counts (B), neutrophil counts (C), and lymphocyte counts (D). Each symbol indicates the values for an individual, with a triangle referring to a person from the rural group, and a square referring to a person from the urban group.

Fig 3: The improvement of reaction time (ms) for the 5 conditions in the Stroop Color Word Task. Values are denoted as mean (SD), * $p < 0.05$, ** $p < 0.01$.

Supplementary table S1. Total duration of the training sessions (minutes) with the respective dates and the duration of walking and running specified.

Figure 1

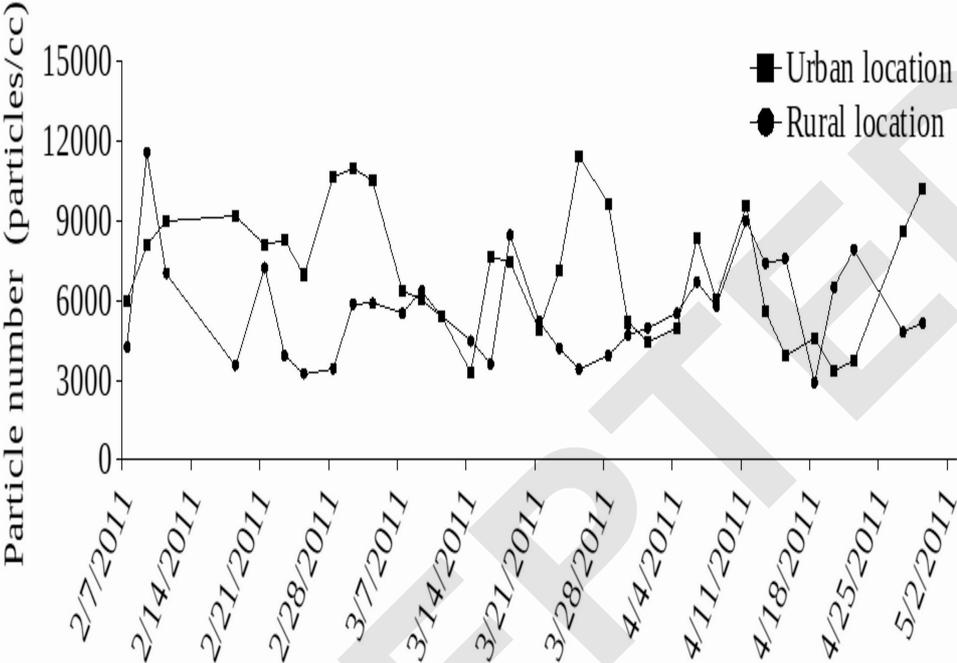


Figure 2

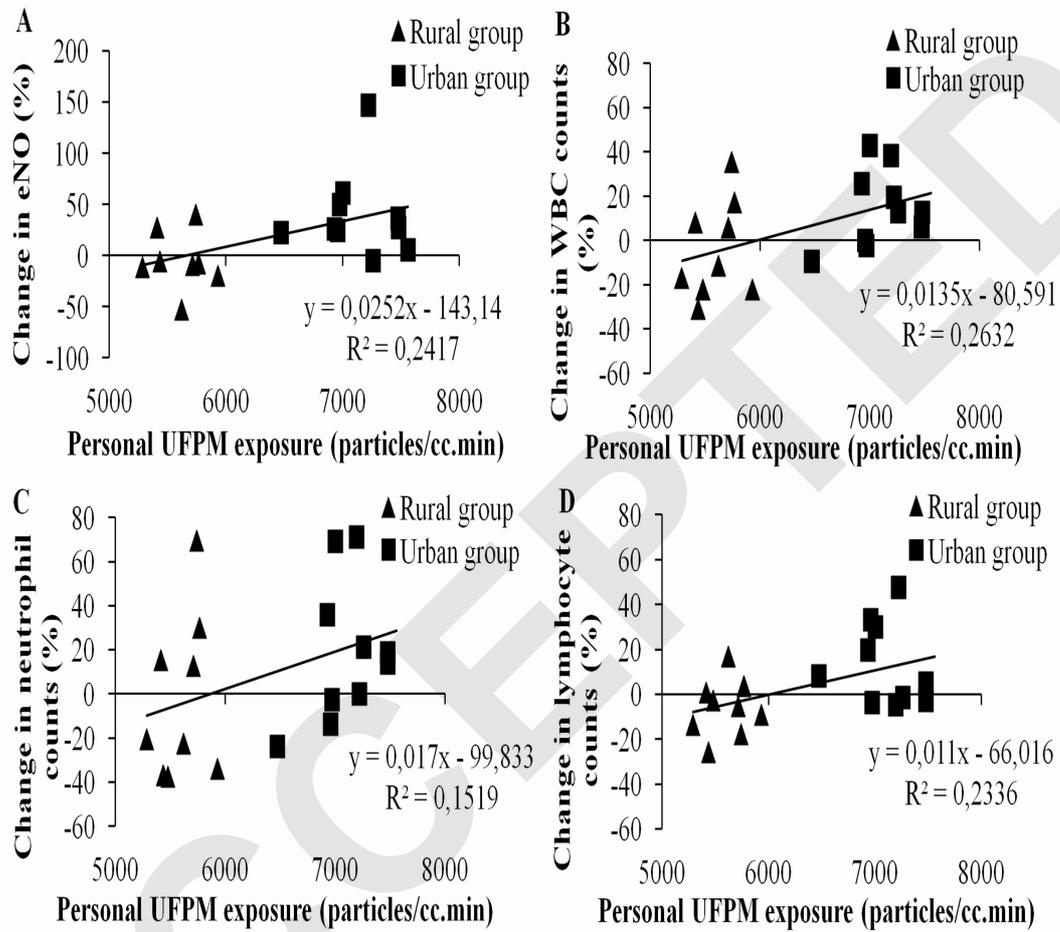


Figure 3

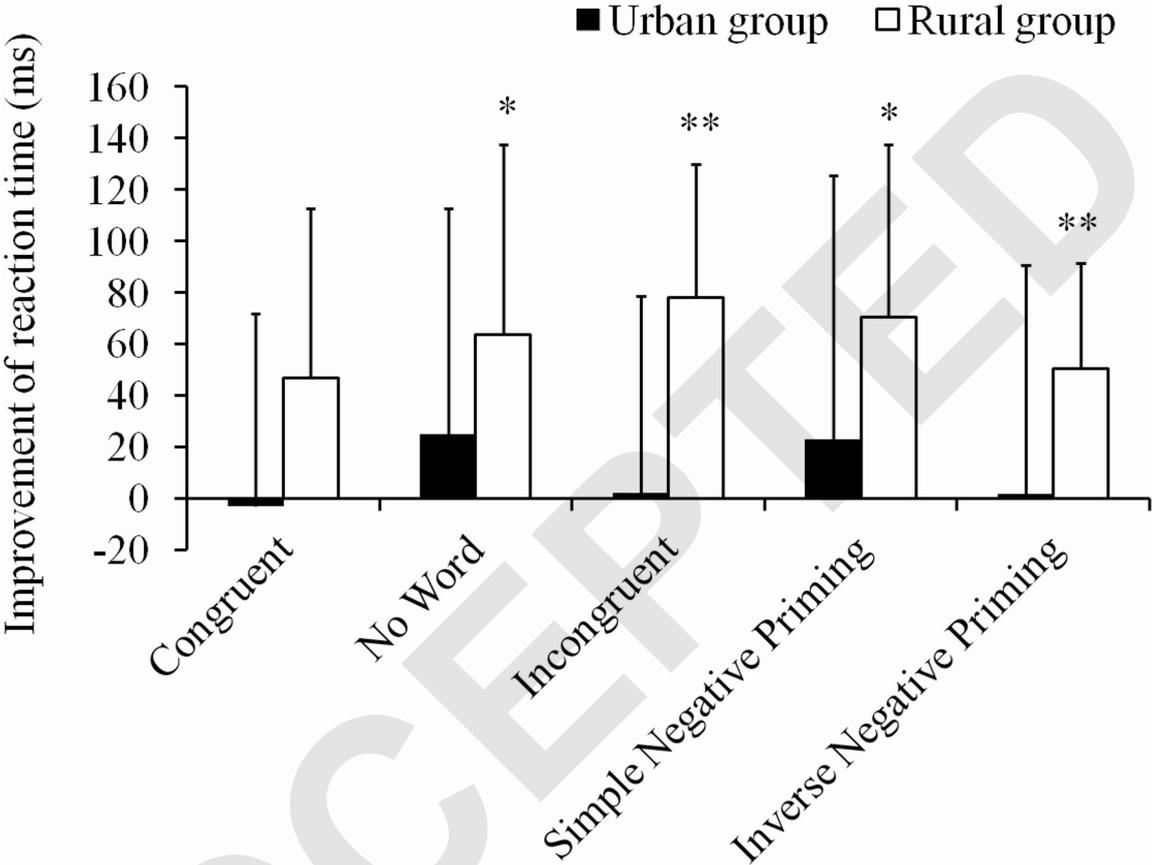


Table 1: Baseline characteristics and measurements of the subjects.

	Urban group	Rural group	<i>P</i>
	(n = 15)	(n = 9)	
Men/Women	4/11	5/4	
Age (years)	28 (8)	39 (6)	<0.001 [#]
BMI (kg.m ⁻²)	24 (0.7)	24 (0.5)	0.51 [§]
Weight (kg)	71 (7)	73 (11)	0.56 [§]
Height (cm)	171 (9)	175 (9)	0.34 [§]
Smokers	2	1	
VO _{2max} (ml.kg ⁻¹ .min ⁻¹)	38.5 (6.2)	37.9 (7)	0.85 [#]
HR _{max} (bmp)	189 (11)	189 (7)	0.68 [#]
Watt _{max}	189 (38)	214 (42)	0.32 [#]

Values are mean (SD) or numbers. P-values denote level of significance between the urban training group and the rural training group. Statistical analyses were performed using [§]independent samples T test or [#]Mann Whitney U Test. BMI, Body Mass Index; HR_{max}, maximal heart rate.

Table 2 Biological measures

	Urban group			Rural group		
	Pre	Post	P	Pre	Post	P
Cooper test performance, m	1864 (297)	2151 (329)	<0.001 [§]	1833 (138)	2225 (274)	<0.001 [§]
sBDNF concentration, ng/ml	29.6 (15.9)	27.7 (12.2)	0.52 [§]	28.0 (5)	26.2 (4.7)	0.08 [§]
Exhaled NO, ppb	16.0 (8.4)	20.6 (10.6)	0.002 [#]	14.5 (4.2)	14.1 (7.5)	0.52 [#]
Blood Leukocyte counts, per μ l	6196 (884)	6967 (1081)	0.02 [§]	6848 (995)	6464 (1292)	0.47 [§]
Blood Neutrophil counts, per μ l	3559 (656)	4147 (1003)	0.04 [§]	3911 (726)	3687 (1226)	0.65 [§]
Blood Lymphocyte counts, per μ l	1996 (412)	2208 (568)	0.08 [§]	2274 (436)	2109 (319)	0.14 [§]
Blood Eosinophil counts, per μ l	156 (93)	116 (76)	0.13 [§]	148 (90)	149 (97)	0.93 [§]
Blood Monocyte counts, per μ l	454 (130)	421 (138)	0.59 [#]	483 (179)	486 (137)	0.84 [#]
Blood Basophil counts, per μ l	30 (14)	32 (23)	0.96 [#]	31 (3)	33 (11)	0.75 [#]

Values are mean (SD). P-values denote level of significance between the measurements pre and post intervention.

Statistical analyses were performed using [§]paired samples T-test or [#]Wilcoxon Signed-Rank Test.

Supplementary table S1. Total duration of the training sessions (minutes) with the respective dates and the duration of walking and running specified.

Date	Total duration (min)	Running time (min)	Walking time (min)
7/02/2011	12*		
9/02/2011	20	10	10
11/02/2011	20	10	10
14/02/2011	20	10	10
16/02/2011	24	12	12
18/02/2011	24	12	12
21/02/2011	24	12	12
23/02/2011	27	14	13
25/02/2011	27	14	13
28/02/2011	27	14	13
2/03/2011	29	17	12
4/03/2011	29	17	12
7/03/2011	29	17	12
9/03/2011	30	18	12
11/03/2011	30	18	12
14/03/2011	30	18	12
16/03/2011	32	24	8
18/03/2011	32	24	8
21/03/2011	32	24	8
23/03/2011	33	26	7
25/03/2011	33	26	7
28/03/2011	33	26	7
30/03/2011	35	30	5
1/04/2011	35	30	5
4/04/2011	35	30	5
6/04/2011	39	34	5
8/04/2011	39	34	5
11/04/2011	39	34	5
13/04/2011	28	26 - 28	0 - 2
15/04/2011	30	26 - 28	0 - 2
18/04/2011	32	30 - 32	0 - 2
20/04/2011	32	30 - 32	0 - 2
22/04/2011	32	30 - 32	0 - 2
27/04/2011	32	32	0
29/04/2011	32	32	0

An asterisk (*) refers to the Cooper test. Detailed information on the Start to Run program can be found on the following website: <http://sport.be.msn.com/starttorun/2009/nl/training/schema05/> (in Dutch).