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Urinary *t,t*-muconic acid as a proxy-biomarker of car exhaust and neurobehavioral performance in 15-year olds

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ABSTRACT

Introduction

Traffic-related air pollution has been shown to induce neurotoxicity in rodents. Several recent epidemiological studies reported negative associations between residential outdoor air pollution and neurobehavioral performance. We investigated in a population of non-smoker adolescents the associations between the urinary concentration of *trans,trans*-muconic acid (*t,t*-MA-U), a metabolite of benzene and used as proxy-biomarker of traffic exposure, and two neurobehavioral domains, i.e. sustained attention and short-term memory.

Methods

In the framework of an environmental health surveillance study in Flanders (Belgium), we examined between 2008 and 2014 grade nine high school students (n = 895). We used reaction time, number of omission errors, and number of commission errors in the Continuous Performance Test to evaluate sustained attention, and for the evaluation of short-term memory we used maximum digit span forward and backward of the Digit Span Test. We measured blood lead (PbB) to assess the independent effect of *t,t*-MA-U on neurobehavioral outcomes.

Results

This neurobehavioral examination study showed that a ten-fold increase in *t,t*-MA-U was associated with a 0.14 SD lower sustained attention (95% Confidence Interval: -0.26 to -0.019; p = 0.02) and a 0.17 SD diminished short-term memory (95% CI: -0.31 to -0.030; p = 0.02). For the same increment in *t,t*-MA-U, the Continuous Performance Test showed a 12.2 msec higher mean reaction time (95% CI: 4.86 to 19.5; p = 0.001) and 0.51 more numbers of errors of omission (95% CI: 0.057 to 0.97; p = 0.028) , while no significant association was found with errors of commission. For the Digit Span Tests, the maximum digit span forward was associated with a 0.20 lower number of digits (95% CI: -0.38 to -0.026; p = 0.025) and maximum digit span backward with -0.15 digits (95% CI: -0.32 to 0.022; p = 0.088). These

associations were independent of PbB, parental education and other important covariates including gender, age, passive smoking, ethnicity, urinary creatinine, time of the day, and examination day of the week. For PbB, an independent association was only found with mean reaction time of the Continuous Performance Test (19.1 msec, 95% CI: 2.43 to 35.8; p = 0.025).

Conclusions

In adolescents, a ten-fold increase in the concentration of *t,t*-MA-U, used as a proxy-biomarker for traffic-related exposure, was associated with a significant deficit in sustained attention and short-term memory. The public health implications of this finding cannot be overlooked as the effect-size for these neurobehavioral domains was about 40% of the effect-size of parental education.

Keywords: traffic-related air pollution, car exhaust, *trans, trans*-muconic acid, blood lead, neurobehavioral performance, adolescents

Abbreviations: *trans, trans*-muconic acid (*t,t*-MA-U), blood lead (PbB), nitrogen dioxide (NO_2), neurobehavioral evaluation system (NES), polycyclic aromatic hydrocarbons (PAHs)

1. Introduction

Historically, traffic was considered a threat to children's mental health because of its contribution to environmental lead contamination (Gilbert and Weiss, 2006). Despite the phase-out of leaded gasoline , a number of recent studies reported negative associations between neurobehavioral outcomes and indicators of traffic-related air pollution exposure in schoolchildren (Chiu et al., 2013; Edwards et al., 2010; Kicinski et al., 2015; Suglia et al., 2008; Wang et al., 2009). These observations from epidemiological research have been supported by experimental studies showing that traffic-related air pollution induces neurobehavioral effects in rodents (Fonken et al., 2011; Suzuki et al., 2010; Zanchi et al., 2010).

The assessment of exposure is a major challenge in human studies involving traffic-related air pollution. It is an important advantage when an epidemiologic investigation can rely on a biomarker of internal exposure that has the potential to reflect the total exposure of an individual. Vehicle traffic is a major source of environmental pollutants including noise, nitrogen oxides, carbon monoxide, black carbon, polycyclic aromatic hydrocarbons, toxic metals, and benzene, a constituent of gasoline. *Trans,trans*-muconic acid (*t,t*-MA), a urinary metabolite of benzene, may be a useful proxy-biomarker of inhalation exposure from traffic as it has the potential of integrating exposures at different locations together with the commute-related exposure. Several studies showed that the urinary concentration of *t,t*-MA (*t,t*-MA-U) strongly increases as a result of exposure to traffic exhausts (Amodio-Cocchieri et al., 2001; Arayasiri et al., 2010; Fustinoni et al., 2005). Furthermore, the usefulness of *t,t*-MA-U as a biomarker of traffic-related benzene exposure in Flemish adolescents has been supported by a study showing that living in suburbs crossed by busy highways (> 80,000 vehicles per day) was associated with higher levels of *t,t*-MA-U in comparison to a control area with little traffic (Staessen et al., 2001).

Here, we report for adolescents the association between two neurobehavioral domains (sustained attention and short-term memory) and traffic-related exposure as reflected by *t,t*-MA-U. The use of a proxy biomarker is unique in the context of the association between cognition and external exposure to traffic. Because environmental lead exposure is well known for its neurotoxic effects particularly in youth, we also included blood lead (PbB) as biomarker of exposure in this epidemiological investigation.

2. Methods

2.1 Study population and data collection

The study was a part of the biomonitoring program for environmental health surveillance in Flanders, Belgium. During the study period, the population-weighted concentrations of traffic-related air pollutants such as NO_2 averaged 30 µg/m³. Between 2008 and 2014, we invited 9th grade high school students (14-15 years old) whose parents were able to fill out a questionnaire in Dutch. The study group was selected from the general Flemish population by random sampling through a multistage sampling design. First, we sampled four schools from each of the five Flemish provinces. Then, we invited students during meetings organized in the schools and participants were sampled from these schools. The number of participants per province was kept proportional to the number of inhabitants in that province. In addition, we recruited also in the municipalities of Genk, Menen, and Gent, as selected hot spots for this human biomonitoring program. In the latter case, addresses of adolescents were obtained from the population registry of the municipalities and we invited the adolescents via a letter sent to their home address. When the desired number of participants was not reached (200 per area), we additionally organized meetings at schools and visited adolescents at home.

Approximately 10 days before the neurobehavioral examination, both adolescents and their parents completed a questionnaire providing information on demographic and lifestyle

characteristics. Additionally, at the day of the neurobehavioral examination, the adolescents filled out a questionnaire by themselves at school without supervision of parents, guardians or teachers in which sensitive information such as their smoking behavior and drug intake was collected (De Craemer et al., 2016). Smokers were excluded from this study because benzene is a constituent of inhaled tobacco smoke resulting in much higher *t,t*-MA-U concentrations in smokers than in non-smokers (Lauwerys and Hoet, 2001). Questions about the socioeconomic status and passive smoking were included in the questionnaire for the parents. Adolescents exposed to secondary tobacco smoke at home were classified as passive smokers. As indicator of the education level of the parents we used the highest education level of either parent (no high school diploma, high school diploma, college or university diploma). Ethnicity was based on parental origin of birth (European, Non-European).

The day before the neurobehavioral examination, each participating adolescent received a plastic bottle and was asked to collect a urine specimen the next morning. The bottles with the urine were stored at school in a cooler (4°C) during the day of examination and afterwards kept frozen at -20°C until analysis. Both parents and their children provided informed consent for participation. The study was approved by the Ethical Committee of the University of Antwerp and complied with the Helsinki declaration.

2.2 Residential outdoor traffic-related exposure estimates

We interpolated the regional background levels of NO_2 for each child's residential address using a spatial temporal interpolation method (Kriging) (Janssen et al., 2008) that uses land cover data obtained from satellite images (CORINE land cover data set) in combination with monitoring stations (n = 44) (Lefebvre et al., 2013; Maiheu et al., 2012). This model chain provided daily interpolated exposure values in a high resolution receptor grid using data from the Belgian telemetric air quality networks, point sources, and line sources. Overall model performance was evaluated by leave-one-out cross-validation based on 44 different locations.

Validation statistics of the interpolation tool showed a temporal explained variance (R^2) > 0.78 for NO_2 (Maiheu et al., 2012). We estimated the exposure up to 30 days before the examinations of the adolescents.

2.3 Internal indicators of traffic-related exposure

The concentrations of *t,t*-MA-U were measured with the method of Angerer et al. (Angerer and Schaller, 1997). Ion-exchange chromatography was used to isolate *t,t*-MA from the urine and after elution with acetic acid (10%) *t,t*-MA was separated from the other components with High Performance Liquid Chromatography and determined with a diode array detector. The urinary concentration of *t,t*-MA was expressed per gram creatinine. Lead concentrations in whole blood (PbB) were measured using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Schroijen et al., 2008).

2.4 Neurobehavioral testing

The Neurobehavioral Evaluation System (NES) is a computerized battery of tests that was developed to study neurobehavioral effects of neurotoxicants in humans (Proctor et al., 2000). Here, we used two tests from the NES3 version of the battery: the Continuous Performance Test (sustained attention domain) and the Digit Span Test (short-term memory domain) (Letz, 2000; White et al., 2003).

In the Continuous Performance Test, letters appeared on the computer screen. The task was to respond as fast as possible to the letter S and not to react to other letters. A new letter is displayed each 1000 msec and remained on the screen for 200 msec. The test consists of five blocks of 12 letters and the last four blocks are used to compute the performance parameters of sustained attention, i.e., the mean reaction time for correct responses, the number of errors of omission (*i.e.*, the number of non-responses) and the numbers of errors of commission (*i.e.*, number of false positive responses).

In the Digit Span Test, the task is to reproduce sequences of digits after an auditory presentation. The test consists of a forward and a backward part. The forward part starts with a sequence of three digits. When a sequence is correctly reproduced, a new sequence is presented consisting of one digit more than the previous one, otherwise a sequence of the same length is presented again. The forward part of the test stops when a subject fails to reproduce two sequences in a row. In the backward condition, the task is to reproduce digits in the reverse order. The maximum numbers of digits correctly reproduced in the forward or backward order were the performance parameters to evaluate short-term memory.

2.5 Statistical analysis

We used SAS software version 9.3 (SAS Institute Inc, Cary, NC) for database management and statistical analysis. We transformed values of *t,t*-MA-U and PbB logarithmically to reduce the skewness of their distributions. To study the possible confounding structure in the data set, we assessed the distributions of continuous variables (ANOVA) and the proportions of categorical variables (χ^2-statistics) across the tertiles of *t,t*-MA-U. To explore the usefulness of *t,t*-MA-U as a proxy for traffic-related exposure, we used a multivariate adjusted linear regression model to study the correlation between *t,t*-MA-U and modeled outdoor residential NO_2 exposure of 72 hours, one week, and one month before the urine collection.

We used multiple regression analysis to study the associations between the internal exposure markers (*i.e.*, *t,t*-MA-U and PbB), and each neurobehavioral parameter separately (*i.e.*, the mean reaction time, the number of errors of omission, and the number of errors of commission in the Continuous Performance Test, and the maximum span forward and backward in the Digit Span Test). In a second analysis, we explored the associations between these internal exposure markers and the neurobehavioral domains, *i.e.*, sustained attention (a combination of the mean reaction time, the number of errors of omission, and the number of

errors of commission for the Continuous Performance Test) and short-term memory (a combination of the maximum span forward and backward for the Digit Span Test). Therefore, the neurobehavioral parameters scores were transformed to z-scores so that all the data will share a common underlying distribution. The combined test parameters were treated as a single outcome for each neurobehavioral domain and were entered into a mixed model. The mixed model adjusts for the correlation between the test performances of a single individual, while differences between the tests are accounted for by entering them as a fixed effect into the model. The assumption that the effects of the internal exposure biomarkers were the same across the neurobehavioral performance tests, was checked by including interaction terms between the tests and the biomarkers. All analyses were adjusted for sex, age, passive smoking, the highest level of education of either parent, ethnicity, urinary creatinine, day of the week, time of the day, and study period, *i.e.,* before or after 2013. The models for *t,t*-MA-U were additionally adjusted for PbB and *vice versa.*

3. Results

3.1 Characteristics of the study group

Table 1 shows data of demographics, exposure, and the neurobehavioral test parameters of the study group consisting of 895 non-smoker adolescents. The mean age was 14.9 years and 460 (51.4%) of the study participants were boys. For 119 (13.3%) of the adolescents, none of the parents had a high school diploma. Most of the adolescents were of European ethnicity (90.8%) and 125 (14.0%) reported to be exposed to passive smoking. The overall geometric mean (range) of PbB was 11.7 (2.7-76.9) µg/L and that of *t,t*-MA-U was 55.3 (1.8-1304) µg/g creatinine. The range of *t,t*-MA-U without standardization for creatinine concentration was 2.5 to 2008 µg/L. Table 1 also shows the participants' characteristics by tertiles of *t,t*-MA-U. The distributions of sex, age, education level of the parents, passive smoking, and time of the

examination did not differ across the *t,t*-MA-U tertiles, while adolescents of non-European origin were more prevalent in the highest *t,t*-MA-U tertiles. In addition, there were more non-European adolescents in the lowest parental socio-economic class (22.7%) than in the higher classes (9.7% and 5.5%) ($p < 0.0001$). The PbB levels and *t,t*-MA-U concentrations correlated significantly with a partial correlation coefficient of 0.17 ($p < 0.0001$).

Table 1. Demographics, exposure, and neurobehavioral data of all participants and by tertiles of the urinary *trans*,*trans*-muconic acid.

		***t*,*t*-MA-U tertiles**			
	All participants N = 895	**1st tertile**	**2nd tertile**	**3rd tertile**	**p-value**
Demographic and lifestyle characteristics					
Boys	460 (51.4%)	159 (34.6%)	144 (31.3%)	157 (34.1%)	0.42
Age (years)	14.88 (0.63)	14.85 (0.61)	14.94 (0.65)	14.86 (0.62)	0.19
Level of education of parents,					0.13
No high school diploma	119 (13.3%)	30 (25.2%)	45 (37.8%)	44 (37.0%)	
High school diploma	289 (32.3%)	89 (30.8%)	102 (35.3%)	98 (33.9%)	
College or university diploma	487 (54.4%)	179 (36.8%)	151 (31.0%)	157 (32.2%)	
Ethnicity					0.007
European	813 (90.8%)	275 (92.3%)	279 (93.6%)	259 (86.6%)	
Passive smoking,	125 (14.0%)	43 (34.4%)	41 (32.8%)	41 (32.8%)	0.96
Examination time,					0.19
Between 8 and 10 am	597 (66.7%)	184 (30.8%)	206 (34.5%)	207 (34.7%)	
Between 10 am and 12 am	223 (24.9%)	88 (39.5%)	70 (31.4%)	65 (29.1%)	
Between 12 and 15 pm	75 (8.4%)	26 (34.7%)	22 (29.3%)	27 (36.0%)	
Exposure measurements					
PbB (µg/L)	11.7 (2.7 – 76.9)	10.8 (2.7 – 38.1)	11.4 (3.3 – 76.9)	12.9 (4.5 – 73.9)	<0.0001
t,*t*-MA-U, µg/g creatinine	55.3 (1.8 – 1304)	22.8 (1.8 – 34)	48.2 (35 – 72)	153.2 (73– 1304)	n.a.
Neurobehavioral tests					
Continuous Performance Test,					
Mean reaction time, msec, (N=832)[a]	415.3 (41.2)	411.7 (38.5)	415.0 (41.3)	419.2 (43.5)	0.10
Errors of omission, count, (N=820)[a]	2.05 (2.56)	1.84 (2.31)	1.80 (2.33)	2.55 (2.96)	0.0006
Errors of commission, count, (N=820)[a]	4.78 (3.19)	4.73 (3.18)	4.59 (3.09)	5.03 (3.29)	0.27
Digit Span Test,					
Maximum span forward, count	5.56 (1.03)	5.57 (0.97)	5.60 (1.03)	5.51 (1.08)	0.55
Maximum span backward, count, (N=881)[a]	4.50 (0.99)	4.53 (0.91)	4.57 (0.97)	4.40 (1.08)	0.10

Count (percent) is given for the categorical variables. Geometric mean (range) is shown for blood lead (PbB) and *trans*,*trans*-muconic acid in urine (*t*,*t*-MA-U). Arithmetic mean (standard deviation) is given for the remaining continuous variables.

[a] Number of participants for whom test results were available.

3.2 Urinary *t,t*-muconic acid as proxy-biomarker for traffic-related exposure

The recent (last 72 hours) residential ambient NO_2 concentration averaged (SD) 22.8 (7.8) µg/m³. A 10 µg/m³ increase in NO_2 during the last 72 hours was significantly associated with a 11.3% increase in urinary *t,t*-MA [95% Confidence Interval (95% CI): 2.87 to 20.5%; p = 0.008], whereas in the same model no significant association was found for distance from school to major road (p = 0.22) or residential proximity to a major road (p = 0.25). The estimates were independent of sex, age, passive smoking, the education level of the parents, and time of sampling. The corresponding estimates for the associations between urinary *t,t*-MA and one week or one month residential NO_2 exposure were 11.4% (95% CI: 2.27 to 21.3%; p = 0.012) and 13.2% (95% CI: 2.07 to 25.6%; p = 0.020) respectively.

3.3 Demographic correlates of neurobehavioral outcomes

Our study showed that the parents' education level was an important predictor of the outcome for the two neurobehavioral domains under study. For adolescents with one of their parents without high school diploma (low parental education level), sustained attention was 0.34 SD lower (95% CI: -0.51 to -0.16; p = 0.0002) and short-term memory 0.44 SD lower (95% CI: -0.61 to -0.28; p < 0.0001) when compared to adolescents whose both parents had a college or university degree (high parental education level) (Table 2). The corresponding results for each test parameter separately are shown in Table 3, i.e. 12.2 msec higher mean reaction time (95% CI: 3.17 to 20.9; p = 0.008), 1.31 more numbers of errors of omission (95% CI: 0.78 to 1.86; p = <0.0001), and 0.70 more number of errors of commission (95% CI: 0.01 to 1.39; p = 0.05) (Continuous Performance Test), and 0.38 less digits for the maximum span forward (95% CI: -0.60 to -0.17; p = 0.0004) and 0.50 less digits for the maximum span backward (95% CI: -0.70 to -0.13; p < 0.0001) (Digit Span test). This parental education-linked association was also shown when comparing medium with high parental education (Tables 2 and 3). Furthermore, sustained attention was lower in boys than girls (-0.19 SD, 95% CI: -0.28 to -

231 0.09; p = 0.0001) and increased with age. For each year-increase in age sustained attention
232 was 0.17 SD (95% CI: 0.10 to 0.24; p < 0.0001) higher. None of the other variables had a
233 significant effect on sustained attention or short-term memory.

234 **Table 2.** Associations of *t,t*-MA-U, PbB, and parental education with
235 neurobehavioral domains.

	Effect size	95% CI	p-value
Sustained attention (N=820)[a]			
t,t-MA-U	-0.14	-0.26 to -0.019	0.02
PbB	-0.22	-0.47 to 0.02	0.07
Parental education			
Low vs high	-0.34	-0.51 to -0.16	0.0002
Medium vs high	-0.17	-0.27 to -0.076	0.0005
Short-term memory (N=881)[a]			
t,t-MA-U	-0.17	-0.31 to -0.030	0.02
PbB	-0.22	-0.52 to 0.080	0.15
Parental education			
Low vs high	-0.44	-0.61 to -0.28	<0.0001
Medium vs high	-0.30	-0.42 to -0.19	<0.0001

CI: confidence intervals.
Effect size is shown for a ten-fold increase in the biomarkers of exposure as reflected by the level of *trans,trans*-muconic acid in urine (*t,t*-MA-U) and blood lead (PbB). Results are expressed as the number of standard deviations change in the neurobehavioral domains. All models allowed for sex, age, passive smoking, parental education level, ethnicity, urinary creatinine, time of the day, day of the week, and study period. The models for *t,t*-MA-U were additionally adjusted for PbB and *vice versa*. The models for parental education were adjusted for the same covariates and the two biomarkers of exposures.
[a] Number of participants for whom test results were available.

236

237 **3.4 Neurobehavioral outcomes in association with *t,t*-MA-U and PbB**

238 In a first analysis, we studied the five neurobehavioral test parameters separately. Before
239 (Figure 1) and after adjustment for covariates and PbB (Table 3), a ten-fold increase in *t,t*-
240 MA-U was significantly associated with a 12.2 msec higher mean reaction time (95% CI: 4.86
241 to 19.5; p = 0.001), 0.51 more numbers of errors of omission (95% CI: 0.057 to 0.97; p =
242 0.028), and not with errors of commission (p = 0.44) (Continuous Performance Test).
243 Maximum digit span forward decreased by 0.20 digits (95% CI: -0.38 to -0.026; p = 0.025)

and maximum digit span backward by 0.15 digits (95% CI: -0.32 to 0.022; p = 0.088) (Digit Span Test). Furthermore, the effect-sizes of t,t-MA-U on these neurobehavioral performances were similar across the different parental education groups, because the interaction-terms were not significant ($p \geq 0.40$). After adjustment for covariates and t,t-MA-U, the associations between these test performances and PbB only reached the level of significance for the mean reaction time in the Continuous Performance Test (19.1 msec, 95% CI: 2.43 to 35.8; p = 0.025). A sensitivity analysis, excluding adolescents of non-European origin did not change the results of the main analyses, except for the association between the mean reaction time (Continuous Performance Test) and PbB (p = 0.025 to p = 0.06).

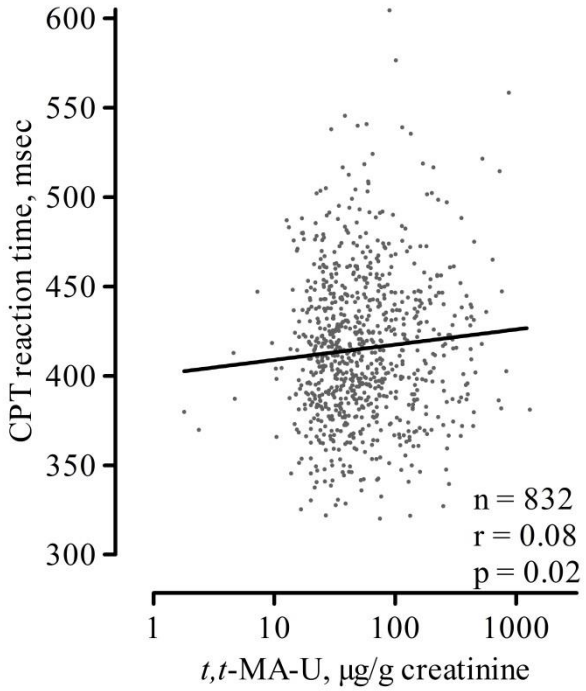

Figure 1 Scatter plot of the mean reaction time in the Continuous Performance Test (CPT) and the urinary levels of *trans,trans*-muconic acid (*t,t*-MA-U).

Table 3. Associations of parental education, *t,t*-MA-U, and PbB with neurobehavioral parameters.

	Parental education				***t,t*-MA-U**		**PbB**	
	Low vs high		**Medium vs high**					
	Effect size (95% CI)	p-value	Effect size (95% CI)	p-value	Effect size (95% CI)	p-value	Effect size (95% CI)	p-value
Continuous Performance Test								
Mean reaction time, msec (N=832)[a]	12.2 (3.17 to 20.9)	0.008	6.70 (0.55 to 12.9)	0.03	12.2 (4.86 to 19.5)	0.001	19.1 (2.43 to 35.8)	0.025
Errors of omission, numbers (N=820)[a]	1.31 (0.78 to 1.86)	<0.0001	0.60 (0.21 to 0.98)	0.002	0.51 (0.057 to 0.97)	0.028	0.25 (-0.78 to 1.29)	0.63
Errors of commission, numbers (N=820)[a]	0.70 (0.01 to 1.39)	0.05	0.41 (-0.07 to 0.89)	0.09	-0.22 (-0.79 to 0.35)	0.44	0.43 (-0.85 to 1.72)	0.51
Digit Span Test								
Max. span forward, digits	-0.38 (-0.60 to -0.17)	0.0004	-0.34 (-0.49 to -0.19)	<0.0001	-0.20 (-0.38 to -0.026)	0.025	-0.30 (-0.70 to 0.098)	0.14
Max. span backward, digits (N=881)[a]	-0.50 (-0.70 to -0.13)	<0.0001	-0.27 (-0.42 to -0.13)	0.0003	-0.15 (-0.32 to 0.022)	0.088	-0.14 (-0.53 to 0.25)	0.48

CI: confidence intervals.

The effect size is shown for a ten-fold increase in the biomarkers of exposure as reflected by the level of *trans,trans*-muconic acid in urine (*t,t*-MA-U) and blood lead (PbB). All models allowed for sex, age, passive smoking, parental education level, ethnicity, urinary creatinine, time of the day, day of the week, and study period. The models for *t,t*-MA-U were additionally adjusted for PbB and *vice versa*. The models for parental education included the same covariates and the two biomarkers of exposure.

[a] Number of participants for whom test results were available.

With regard to the neurobehavioral domains (Table 2), a ten-fold increase in *t,t*-MA-U was associated with a lower sustained attention (-0.14 SD, 95% CI: -0.26 to -0.019; p = 0.02) and lower short-term memory (-0.17 SD, 95% CI: -0.31 to -0.030; p = 0.02). The effect estimates for PbB were in the same direction but did not reach the level of significance. Interaction tests between separate test performances and exposure biomarkers were not significant which supports the assumption of a similar pattern of dose-response for the neurobehavioral parameters of tests which were combined.

4. Discussion

In Europe and the USA, the phase out of lead in gasoline entailed a drastic decrease of the PbB levels in the population at large. Nevertheless, reports of compromised neurobehavioral performances in children still continue to keep our attention focused on traffic-linked public health issues. In the present study, including more than 800 non-smoker adolescents, we found evidence of inverse associations between a proxy-biomarker of traffic-related exposure, *i.e., t,t*-MA-U, and neurobehavioral test performances which were independent of PbB and other covariates studied. For example, a ten-fold increase in the level of *t,t*-MA-U was associated with a 0.14 SD decrease in sustained attention (95% CI: -0.26 to -0.019) and a 0.17 SD decrease in short-term memory (95% CI: -0.31 to -0.030). The public health significance of these estimates is striking when we compare them with our estimates of low *versus* high parental education. This comparison revealed that traffic-related air pollution, as reflected by *t,t*-MA-U, has an effect size corresponding to 41% of the effect size for parental education on sustained attention and 39% of the effect size for parental education on short-term memory. By assessing personal exposure through an internal biomarker related to car exhaust, the current study may offer new perspectives for risk assessment of cognitive function changes in children in relation to traffic-related outdoor air pollution.

Recently, several studies reported a negative association between traffic-related air pollution exposure and neurobehavioral outcomes. In a study of 202 children from Boston with a mean age of 9.7 years, the lifetime residential black carbon concentrations were associated with a decrease in intelligence (Suglia et al., 2008) and a decrease in sustained attention as assessed by the Continuous Performance Test (Chiu et al., 2013). In a Chinese study of 928 nine-year-old children, those living in an area with high concentrations of ambient particulate matter and NO_2 showed poorer performance for a number of cognitive domains including sustained attention assessed by the Continuous Performance Test in comparison to those living in a clean area (Wang et al., 2009). Perinatal exposure to airborne polycyclic aromatic hydrocarbons (PAHs) was associated with a lower intelligence at age five (Edwards et al., 2010; Perera et al., 2009). In the same cohort, an inverse association between perinatal exposure to PAHs and the white matter surface of the brain has been reported recently (Peterson et al., 2015). Observational human studies are supported by the findings of experimental animal research. Mice exposed to environmentally relevant concentrations of ambient $PM_{2.5}$ over a period of 9 months, starting at four weeks of age, showed poorer spatial memory than control animals (Fonken et al., 2011). Another study observed a negative effect of a long-term $PM_{2.5}$ exposure on discriminative memory in rats (Zanchi et al., 2010). Our observation of a negative association between *t,t*-MA-U and neurobehavioral test performances in Flemish adolescents adds to the body of evidence suggesting that traffic-related air pollution exposure negatively affects cognitive functioning.

Like most solvents, benzene rapidly crosses the blood-brain barrier. Following acute inhalation of benzene at doses ranging from 300 to 3,000 ppm, humans exhibit manifestations of central nervous system toxicity, including headache, nausea, tiredness, dizziness, narcosis, and loss of consciousness. These symptoms are reversible when symptomatic workers are removed from the problem area (ATSDR, 2007). To prevent hematotoxic and carcinogenic

288 health effects in exposed workers, the American Conference of Governmental Industrial
289 Hygienists set in 1996 the threshold limit value (TLV, time-weighted-average) for benzene at
290 the stringent level of 0.5 ppm (1.6 mg/m³) (ACGIH, 2016). For comparison, at 17 sites
291 monitoring the quality of ambient air in Flanders, the annual averages of benzene levels
292 ranged in 2011 from 0.53 to 1.97 µg/m³(VMM, 2012), which is more than 800 times lower
293 than the TLV for the industrial setting. Currently, there is no evidence that such a low
294 environmental exposure to benzene may cause neurotoxic effects. Benzene may account for 3
295 to 15 % of the total tailpipe hydrocarbon composition (ATSDR, 2007). Vehicle exhaust is
296 considered the largest anthropogenic source of environmental exposure to benzene and
297 estimated to contribute for 70 to 80% to the overall man-made benzene emissions (ATSDR,
298 2007). Because benzene-linked neurotoxic effects are not likely to occur as a result of traffic
299 exposure and because exposure to traffic-related benzene is accompanied by exposure to other
300 pollutants including nitrogen oxides, carbon monoxide, black carbon, polycyclic aromatic
301 hydrocarbons, toxic metals, and noise, we considered *t,t*-MA-U as a proxy-biomarker of the
302 overall traffic exposure and not as a mere biomarker of benzene exposure. The concentrations
303 of *t,t*-MA-U ranged from 3 to 2008 µg/L in our group of non-smoker adolescents, which is
304 relatively high in light of studies that investigated the levels of *t,t*-MA-U in populations
305 occupationally exposed to traffic. In Genoa (Italy), the concentrations of *t,t*-MA-U ranged
306 from <10 to 2014 µg/L among non-smoker bus drivers and from <10 to 398 µg/L among non-
307 smoker referents (Fustinoni et al., 2005). In Milan, the *t,t*-MA-U range was <10 to 1400 µg/L
308 among non-smoker traffic policemen and <10 to 576 µg/L among referents (Fustinoni et al.,
309 2005). The large between-subject differences, the relatively high concentrations of *t,t*-MA-U,
310 and the substantial correlation with NO_2 levels in ambient air support the reliability of this
311 metabolite of benzene as a proxy-biomarker of traffic exposure in our study.

Several mechanisms of the neurotoxic effects of traffic-related air pollution have been investigated. In rodents, it has been shown that ultrafine particles may translocate by retro-axonal transport via the olfactory nerve to other regions of the brain (Elder et al., 2006; Oberdörster et al., 2004). Moreover, fine particles translocate from the lungs into the blood from which they can reach the brain by crossing the blood-brain barrier (Furuyama et al., 2009; Oberdorster et al., 2002). Examination of the brains of individuals who died suddenly and resided in cities with much air pollution revealed the presence of ultrafine particles in cerebral tissue (Calderon-Garciduenas et al., 2010; Calderon-Garciduenas et al., 2008). The presence of particles in the brain may cause a number of effects including microglial activation (Block et al., 2004), oxidative stress (Davis et al., 2013; Gillespie et al., 2013; Hartz et al., 2008), pro-inflammatory cytokine response (Hartz et al., 2008), neuronal death (Block et al., 2004; Gillespie et al., 2013), and changes in neurotransmission (Davis et al., 2013). Besides these direct effects, release of cytokines from the lungs and translocation of ultrafine particles into the circulation may trigger a sequence of pro-inflammatory events including stimulation of production of leucocytes and platelets in the bone marrow and activation of the vascular endothelium (Hogg and van Eeden, 2009; Van Eeden et al., 2001). Such a systemic response may also affect the brain (Clark et al., 2010; Cunningham, 2013). Studies in rodents exposed to air pollution showed changes in the level and turnover of neurotransmitters (Suzuki et al., 2010; Yokota et al., 2009) and gene expression related to endocrine function (Tsukue et al., 2009; Win-Shwe et al., 2012), increased level of oxidative stress (van Berlo et al., 2010; Zanchi et al., 2010), and a pro-inflammatory cytokine response (Fonken et al., 2011; Gerlofs-Nijland et al., 2010; Win-Shwe et al., 2012). Studies investigating the brains of humans who died suddenly revealed an association between the level of air pollution and the severity of inflammation in the brain (Calderon-Garciduenas et al., 2004; Calderon-Garciduenas et al., 2008). Recently, it has been shown that the placenta

plays a role in neurodevelopmental processes through adaptive responses to the maternal environment (Zeltser and Leibel, 2011). Placental gene expression of Brain-derived neurotrophic factor and Synapsin 1 involved in neurodevelopmental trajectories were inversely associated with $PM_{2.5}$ exposure levels in the first trimester of pregnancy (Saenen et al., 2015).

Our study has several strengths. First, due to the use of *t,t*-MA-U as an individual internal biomarker of exposure, our traffic-related exposure assessment was able to integrate the exposures at different locations with the commute-related exposure. Second, urinary *t,t*-MA was significantly associated with recent external residential NO_2 exposure, a known indicator for ambient traffic-related exposure. Other strengths are the use of an internal proxy-biomarker of personal traffic exposure, the *a priori* exclusion of smokers, and accounting for blood lead with respect to its neurotoxic potential.

The main limitation of our study is its observational character, which involves the risk of confounding by predictors of neurobehavioral performance that may be associated with *t,t*-MA-U. The distributions of the education level of the parents and passive smoking were similar among the participants with low and high *t,t*-MA-U concentrations. This suggests that socioeconomic status and passive smoking were irrelevant as to the associations between *t,t*-MA-U and neurobehavioral test performances. In contrast, adolescents from non-European origin were more prevalent in the highest *t,t*-MA-U tertile. However, excluding adolescents from non-European origin from the main analysis did not alter our findings.

The associations involving traffic-related air pollution, as reflected by *t,t*-MA-U, would most likely benefit from the use of urinary S-phenylmercapturic acid as proxy-biomarker of environmental benzene exposure, because of the higher specificity of this benzene metabolite. Nevertheless, we found *t,t*-MA-U correlating with the modeled residential NO_2 concentration, a well-known proxy for traffic-related air pollution. Self-

reported tobacco use among young people can underestimate the actual prevalence of tobacco use, which might be a limitation of our study. However, the questionnaire was individually filled out by the adolescents at school during the day of the neurobehavioral examination, thus without any potential supervision of parents, guardians, or teachers. In addition, we previously validated the questionnaire by measuring urinary cotinine values which confirms the reliability of the adolescents' self-reported tobacco use when the questionnaire is administered individually (Staessen et al., 2001). Another potential limitation may be the lack of information of hours of sleep in the days previous to the examination.

5. Conclusion

Traffic exposure in adolescents, as reflected by the concentrations of *t,t*-MA-U, a metabolite of benzene in urine, was associated with a lowering of sustained attention (reaction time) and short-term memory. These associations were independent from environmental lead exposure and various other factors among them parental education. The public health implications are evident by showing that for a 10-fold increase of *t,t*-MA-U the estimates of the effect size for sustained attention and short-term memory were about 40% of the effect-size of parental education which is a well-accepted determinant of cognitive function.

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Competing Interests

The authors declare that they have no conflict of interest.

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