

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

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

32 ***Introduction***

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

39 ***Methods***

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

46 ***Results***

47 This neurobehavioral examination study showed that a ten-fold increase in *t,t*-MA-U was
48 associated with a 0.14 SD lower sustained attention (95% Confidence Interval: -0.26 to -
49 0.019; p = 0.02) and a 0.17 SD diminished short-term memory (95% CI: -0.31 to -0.030; p =
50 0.02). For the same increment in *t,t*-MA-U, the Continuous Performance Test showed a 12.2
51 msec higher mean reaction time (95% CI: 4.86 to 19.5; p = 0.001) and 0.51 more numbers of
52 errors of omission (95% CI: 0.057 to 0.97; p = 0.028) , while no significant association was
53 found with errors of commission. For the Digit Span Tests, the maximum digit span forward
54 was associated with a 0.20 lower number of digits (95% CI: -0.38 to -0.026; p = 0.025) and
55 maximum digit span backward with -0.15 digits (95% CI: -0.32 to 0.022; p = 0.088). These

56 associations were independent of PbB, parental education and other important covariates
57 including gender, age, passive smoking, ethnicity, urinary creatinine, time of the day, and
58 examination day of the week. For PbB, an independent association was only found with mean
59 reaction time of the Continuous Performance Test (19.1 msec, 95% CI: 2.43 to 35.8; p =
60 0.025).

61 **Conclusions**

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

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

69

70 **Abbreviations:** *trans*, *trans*-muconic acid (*t,t*-MA-U), blood lead (PbB), nitrogen dioxide
71 (NO₂), neurobehavioral evaluation system (NES), polycyclic aromatic hydrocarbons (PAHs)

72 **1. Introduction**

73 Historically, traffic was considered a threat to children's mental health because of its
74 contribution to environmental lead contamination (Gilbert and Weiss, 2006). Despite the
75 phase-out of leaded gasoline, a number of recent studies reported negative associations
76 between neurobehavioral outcomes and indicators of traffic-related air pollution exposure in
77 schoolchildren (Chiu et al., 2013; Edwards et al., 2010; Kicinski et al., 2015; Suglia et al.,
78 2008; Wang et al., 2009). These observations from epidemiological research have been
79 supported by experimental studies showing that traffic-related air pollution induces
80 neurobehavioral effects in rodents (Fonken et al., 2011; Suzuki et al., 2010; Zanchi et al.,
81 2010).

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

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

103 **2. Methods**

104 **2.1 Study population and data collection**

105 The study was a part of the biomonitoring program for environmental health surveillance in
106 Flanders, Belgium. During the study period, the population-weighted concentrations of
107 traffic-related air pollutants such as NO₂ averaged 30 µg/m³. Between 2008 and 2014, we
108 invited 9th grade high school students (14-15 years old) whose parents were able to fill out a
109 questionnaire in Dutch. The study group was selected from the general Flemish population by
110 random sampling through a multistage sampling design. First, we sampled four schools from
111 each of the five Flemish provinces. Then, we invited students during meetings organized in
112 the schools and participants were sampled from these schools. The number of participants per
113 province was kept proportional to the number of inhabitants in that province. In addition, we
114 recruited also in the municipalities of Genk, Menen, and Gent, as selected hot spots for this
115 human biomonitoring program. In the latter case, addresses of adolescents were obtained from
116 the population registry of the municipalities and we invited the adolescents via a letter sent to
117 their home address. When the desired number of participants was not reached (200 per area),
118 we additionally organized meetings at schools and visited adolescents at home.

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

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

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

138 **2.2 Residential outdoor traffic-related exposure estimates**

139 We interpolated the regional background levels of NO₂ for each child's residential address
140 using a spatial temporal interpolation method (Kriging) (Janssen et al., 2008) that uses land
141 cover data obtained from satellite images (CORINE land cover data set) in combination with
142 monitoring stations (n = 44) (Lefebvre et al., 2013; Maiheu et al., 2012). This model chain
143 provided daily interpolated exposure values in a high resolution receptor grid using data from
144 the Belgian telemetric air quality networks, point sources, and line sources. Overall model
145 performance was evaluated by leave-one-out cross-validation based on 44 different locations.

146 Validation statistics of the interpolation tool showed a temporal explained variance (R^2) >
147 0.78 for NO₂ (Maiheu et al., 2012). We estimated the exposure up to 30 days before the
148 examinations of the adolescents.

149 **2.3 Internal indicators of traffic-related exposure**

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

157 **2.4 Neurobehavioral testing**

158 The Neurobehavioral Evaluation System (NES) is a computerized battery of tests that was
159 developed to study neurobehavioral effects of neurotoxicants in humans (Proctor et al., 2000).
160 Here, we used two tests from the NES3 version of the battery: the Continuous Performance
161 Test (sustained attention domain) and the Digit Span Test (short-term memory domain) (Letz,
162 2000; White et al., 2003).

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

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

178 **2.5 Statistical analysis**

179 We used SAS software version 9.3 (SAS Institute Inc, Cary, NC) for database management
180 and statistical analysis. We transformed values of *t,t*-MA-U and PbB logarithmically to
181 reduce the skewness of their distributions. To study the possible confounding structure in the
182 data set, we assessed the distributions of continuous variables (ANOVA) and the proportions
183 of categorical variables (χ^2 -statistics) across the tertiles of *t,t*-MA-U. To explore the
184 usefulness of *t,t*-MA-U as a proxy for traffic-related exposure, we used a multivariate
185 adjusted linear regression model to study the correlation between *t,t*-MA-U and modeled
186 outdoor residential NO₂ exposure of 72 hours, one week, and one month before the urine
187 collection.

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

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

208 **3. Results**

209 **3.1 Characteristics of the study group**

210 Table 1 shows data of demographics, exposure, and the neurobehavioral test parameters of the
211 study group consisting of 895 non-smoker adolescents. The mean age was 14.9 years and 460
212 (51.4%) of the study participants were boys. For 119 (13.3%) of the adolescents, none of the
213 parents had a high school diploma. Most of the adolescents were of European ethnicity
214 (90.8%) and 125 (14.0%) reported to be exposed to passive smoking. The overall geometric
215 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
216 creatinine. The range of *t,t*-MA-U without standardization for creatinine concentration was
217 2.5 to 2008 µg/L. Table 1 also shows the participants' characteristics by tertiles of *t,t*-MA-U.
218 The distributions of sex, age, education level of the parents, passive smoking, and time of the

219 examination did not differ across the *t,t*-MA-U tertiles, while adolescents of non-European
220 origin were more prevalent in the highest *t,t*-MA-U tertiles. In addition, there were more non-
221 European adolescents in the lowest parental socio-economic class (22.7%) than in the higher
222 classes (9.7% and 5.5%) ($p < 0.0001$). The PbB levels and *t,t*-MA-U concentrations correlated
223 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.

	All participants N = 895	<i>t,t</i> -MA-U tertiles			p-value
		1st tertile	2nd tertile	3rd tertile	
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
<i>t,t</i> -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.

206 **3.2 Urinary *t,t*-muconic acid as proxy-biomarker for traffic-related exposure**

207 The recent (last 72 hours) residential ambient NO₂ concentration averaged (SD) 22.8 (7.8)
208 µg/m³. A 10 µg/m³ increase in NO₂ during the last 72 hours was significantly associated with
209 a 11.3% increase in urinary *t,t*-MA [95% Confidence Interval (95% CI): 2.87 to 20.5%; p =
210 0.008], whereas in the same model no significant association was found for distance from
211 school to major road (p = 0.22) or residential proximity to a major road (p = 0.25). The
212 estimates were independent of sex, age, passive smoking, the education level of the parents,
213 and time of sampling. The corresponding estimates for the associations between urinary *t,t*-
214 MA and one week or one month residential NO₂ exposure were 11.4% (95% CI: 2.27 to
215 21.3%; p = 0.012) and 13.2% (95% CI: 2.07 to 25.6%; p = 0.020) respectively.

216 **3.3 Demographic correlates of neurobehavioral outcomes**

217 Our study showed that the parents' education level was an important predictor of the outcome
218 for the two neurobehavioral domains under study. For adolescents with one of their parents
219 without high school diploma (low parental education level), sustained attention was 0.34 SD
220 lower (95% CI: -0.51 to -0.16; p = 0.0002) and short-term memory 0.44 SD lower (95% CI: -
221 0.61 to -0.28; p < 0.0001) when compared to adolescents whose both parents had a college or
222 university degree (high parental education level) (Table 2). The corresponding results for each
223 test parameter separately are shown in Table 3, i.e. 12.2 msec higher mean reaction time (95%
224 CI: 3.17 to 20.9; p = 0.008), 1.31 more numbers of errors of omission (95% CI: 0.78 to 1.86;
225 p = <0.0001), and 0.70 more number of errors of commission (95% CI: 0.01 to 1.39; p = 0.05)
226 (Continuous Performance Test), and 0.38 less digits for the maximum span forward (95% CI:
227 -0.60 to -0.17; p = 0.0004) and 0.50 less digits for the maximum span backward (95% CI: -
228 0.70 to -0.13; p < 0.0001) (Digit Span test). This parental education-linked association was
229 also shown when comparing medium with high parental education (Tables 2 and 3).
230 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			
<i>t,t</i> -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			
<i>t,t</i> -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.

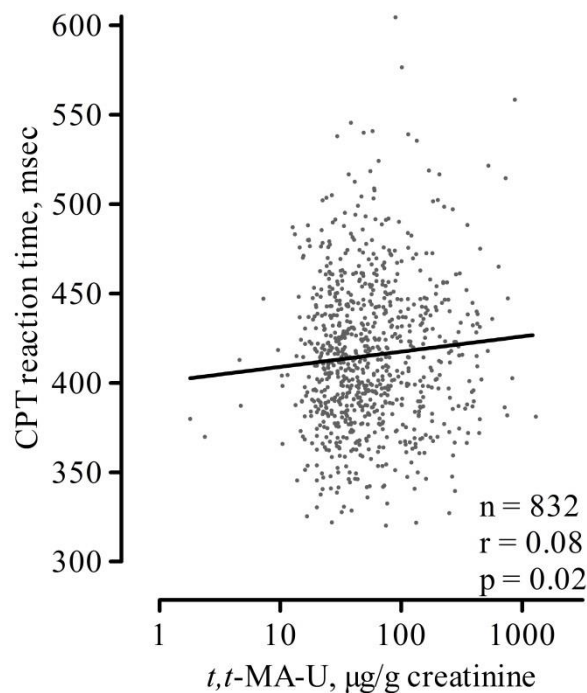
^a Number of participants for whom test results were available.

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; $p = 0.025$)

244 and maximum digit span backward by 0.15 digits (95% CI: -0.32 to 0.022; $p = 0.088$) (Digit
245 Span Test). Furthermore, the effect-sizes of *t,t*-MA-U on these neurobehavioral performances
246 were similar across the different parental education groups, because the interaction-terms
247 were not significant ($p \geq 0.40$). After adjustment for covariates and *t,t*-MA-U, the associations
248 between these test performances and PbB only reached the level of significance for the mean
249 reaction time in the Continuous Performance Test (19.1 msec, 95% CI: 2.43 to 35.8; $p =$
250 0.025). A sensitivity analysis, excluding adolescents of non-European origin did not change
251 the results of the main analyses, except for the association between the mean reaction time
252 (Continuous Performance Test) and PbB ($p = 0.025$ to $p = 0.06$).
253



254

255 **Figure 1** Scatter plot of the mean reaction time in the Continuous Performance Test (CPT)
256 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				<i>t,t</i> -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.

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

246 **4. Discussion**

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

263 Recently, several studies reported a negative association between traffic-related air
264 pollution exposure and neurobehavioral outcomes. In a study of 202 children from Boston
265 with a mean age of 9.7 years, the lifetime residential black carbon concentrations were
266 associated with a decrease in intelligence (Suglia et al., 2008) and a decrease in sustained
267 attention as assessed by the Continuous Performance Test (Chiu et al., 2013). In a Chinese
268 study of 928 nine-year-old children, those living in an area with high concentrations of
269 ambient particulate matter and NO₂ showed poorer performance for a number of cognitive
270 domains including sustained attention assessed by the Continuous Performance Test in
271 comparison to those living in a clean area (Wang et al., 2009). Perinatal exposure to airborne
272 polycyclic aromatic hydrocarbons (PAHs) was associated with a lower intelligence at age five
273 (Edwards et al., 2010; Perera et al., 2009). In the same cohort, an inverse association between
274 perinatal exposure to PAHs and the white matter surface of the brain has been reported
275 recently (Peterson et al., 2015). Observational human studies are supported by the findings of
276 experimental animal research. Mice exposed to environmentally relevant concentrations of
277 ambient PM_{2.5} over a period of 9 months, starting at four weeks of age, showed poorer spatial
278 memory than control animals (Fonken et al., 2011). Another study observed a negative effect
279 of a long-term PM_{2.5} exposure on discriminative memory in rats (Zanchi et al., 2010). Our
280 observation of a negative association between *t,t*-MA-U and neurobehavioral test
281 performances in Flemish adolescents adds to the body of evidence suggesting that traffic-
282 related air pollution exposure negatively affects cognitive functioning.

283 Like most solvents, benzene rapidly crosses the blood-brain barrier. Following acute
284 inhalation of benzene at doses ranging from 300 to 3,000 ppm, humans exhibit manifestations
285 of central nervous system toxicity, including headache, nausea, tiredness, dizziness, narcosis,
286 and loss of consciousness. These symptoms are reversible when symptomatic workers are
287 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 NO₂ levels in ambient air support the reliability of this
311 metabolite of benzene as a proxy-biomarker of traffic exposure in our study.

312 Several mechanisms of the neurotoxic effects of traffic-related air pollution have been
313 investigated. In rodents, it has been shown that ultrafine particles may translocate by retro-
314 axonal transport via the olfactory nerve to other regions of the brain (Elder et al., 2006;
315 Oberdörster et al., 2004). Moreover, fine particles translocate from the lungs into the blood
316 from which they can reach the brain by crossing the blood-brain barrier (Furuyama et al.,
317 2009; Oberdorster et al., 2002). Examination of the brains of individuals who died suddenly
318 and resided in cities with much air pollution revealed the presence of ultrafine particles in
319 cerebral tissue (Calderon-Garciduenas et al., 2010; Calderon-Garciduenas et al., 2008). The
320 presence of particles in the brain may cause a number of effects including microglial
321 activation (Block et al., 2004), oxidative stress (Davis et al., 2013; Gillespie et al., 2013;
322 Hartz et al., 2008), pro-inflammatory cytokine response (Hartz et al., 2008), neuronal death
323 (Block et al., 2004; Gillespie et al., 2013), and changes in neurotransmission (Davis et al.,
324 2013). Besides these direct effects, release of cytokines from the lungs and translocation of
325 ultrafine particles into the circulation may trigger a sequence of pro-inflammatory events
326 including stimulation of production of leucocytes and platelets in the bone marrow and
327 activation of the vascular endothelium (Hogg and van Eeden, 2009; Van Eeden et al., 2001).
328 Such a systemic response may also affect the brain (Clark et al., 2010; Cunningham, 2013).
329 Studies in rodents exposed to air pollution showed changes in the level and turnover of
330 neurotransmitters (Suzuki et al., 2010; Yokota et al., 2009) and gene expression related to
331 endocrine function (Tsukue et al., 2009; Win-Shwe et al., 2012), increased level of oxidative
332 stress (van Berlo et al., 2010; Zanchi et al., 2010), and a pro-inflammatory cytokine response
333 (Fonken et al., 2011; Gerlofs-Nijland et al., 2010; Win-Shwe et al., 2012). Studies
334 investigating the brains of humans who died suddenly revealed an association between the
335 level of air pollution and the severity of inflammation in the brain (Calderon-Garciduenas et
336 al., 2004; Calderon-Garciduenas et al., 2008). Recently, it has been shown that the placenta

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

342 Our study has several strengths. First, due to the use of *t,t*-MA-U as an individual
343 internal biomarker of exposure, our traffic-related exposure assessment was able to integrate
344 the exposures at different locations with the commute-related exposure. Second, urinary *t,t*-
345 MA was significantly associated with recent external residential NO₂ exposure, a known
346 indicator for ambient traffic-related exposure. Other strengths are the use of an internal proxy-
347 biomarker of personal traffic exposure, the *a priori* exclusion of smokers, and accounting for
348 blood lead with respect to its neurotoxic potential.

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

357 The associations involving traffic-related air pollution, as reflected by *t,t*-MA-U,
358 would most likely benefit from the use of urinary S-phenylmercapturic acid as proxy-
359 biomarker of environmental benzene exposure, because of the higher specificity of this
360 benzene metabolite. Nevertheless, we found *t,t*-MA-U correlating with the modeled
361 residential NO₂ concentration, a well-known proxy for traffic-related air pollution. Self-

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

370 **5. Conclusion**

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

378

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386 **Competing Interests**

387 The authors declare that they have no conflict of interest.

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Reference List

ACGIH, 2016. TLVs and BEIs. Cincinnati, OH, USA. American Conference of Governmental Industrial Hygienists.

Amodio-Cocchieri, R., et al., 2001. Evaluation of benzene exposure in children living in Campania (Italy) by urinary trans,trans-muconic acid assay. *J Toxicol Environ Health A*. 63, 79-87.

Angerer, J., Schaller, K., 1997. Analyses of hazardous substances in biological materials. Wiley-VCH Verlag, Weinheim.

Arayasiri, M., et al., 2010. Biomonitoring of benzene and 1,3-butadiene exposure and early biological effects in traffic policemen. *Sci Total Environ*. 408, 4855-4862.

ATSDR, 2007. Toxicological profile for benzene. Public Health Services, U.S. Department of Health and Human Services, Atlanta, GA, Agency for Toxic Substances and Disease Registry.

Block, M. L., et al., 2004. Nanometer size diesel exhaust particles are selectively toxic to dopaminergic neurons: the role of microglia, phagocytosis, and NADPH oxidase. *FASEB J*. 18, 1618-1620.

Calderon-Garciduenas, L., et al., 2010. Urban air pollution: influences on olfactory function and pathology in exposed children and young adults. *Exp Toxicol Pathol*. 62, 91-102.

Calderon-Garciduenas, L., et al., 2004. Brain inflammation and Alzheimer's-like pathology in individuals exposed to severe air pollution. *Toxicol Pathol*. 32, 650-8.

Calderon-Garciduenas, L., et al., 2008. Long-term air pollution exposure is associated with neuroinflammation, an altered innate immune response, disruption of the blood-brain barrier, ultrafine particulate deposition, and accumulation of amyloid beta-42 and alpha-synuclein in children and young adults. *Toxicol Pathol*. 36, 289-310.

Chiu, Y. H., et al., 2013. Associations between traffic-related black carbon exposure and attention in a prospective birth cohort of urban children. *Environ Health Perspect*. 121, 859-864.

Clark, I. A., et al., 2010. The roles of TNF in brain dysfunction and disease. *Pharmacol Ther*. 128, 519-548.

Cunningham, C., 2013. Microglia and neurodegeneration: the role of systemic inflammation. *Glia*. 61, 71-90.

Davis, D. A., et al., 2013. Urban air pollutants reduce synaptic function of CA1 neurons via an NMDA/NO pathway in vitro. *J Neurochem*. 127, 509-19.

- 424 De Craemer, S., et al., 2016. Investigating unmetabolized polycyclic aromatic
425 hydrocarbons in adolescents' urine as biomarkers of environmental exposure.
426 *Chemosphere*. 155, 48-56.
- 427 Edwards, S. C., et al., 2010. Prenatal exposure to airborne polycyclic aromatic
428 hydrocarbons and children's intelligence at 5 years of age in a prospective cohort study
429 in Poland. *Environ Health Perspect*. 118, 1326-1331.
- 430 Elder, A., et al., 2006. Translocation of inhaled ultrafine manganese oxide particles to the
431 central nervous system. *Environ Health Perspect*. 114, 1172-1178.
- 432 Fonken, L. K., et al., 2011. Air pollution impairs cognition, provokes depressive-like
433 behaviors and alters hippocampal cytokine expression and morphology. *Mol*
434 *Psychiatry*. 16, 987-95, 973.
- 435 Furuyama, A., et al., 2009. Extrapulmonary translocation of intratracheally instilled fine
436 and ultrafine particles via direct and alveolar macrophage-associated routes. *Arch*
437 *Toxicol*. 83, 429-37.
- 438 Fustinoni, S., et al., 2005. Monitoring low benzene exposure: comparative evaluation of
439 urinary biomarkers, influence of cigarette smoking, and genetic polymorphisms.
440 *Cancer Epidemiol Biomarkers Prev*. 14, 2237-44.
- 441 Gerlofs-Nijland, M. E., et al., 2010. Effect of prolonged exposure to diesel engine exhaust
442 on proinflammatory markers in different regions of the rat brain. Part *Fibre Toxicol*. 7,
443 12.
- 444 Gilbert, S. G., Weiss, B., 2006. A rationale for lowering the blood lead action level from
445 10 to 2 microg/dL. *Neurotoxicology*. 27, 693-701.
- 446 Gillespie, P., et al., 2013. Particulate matter neurotoxicity in culture is size-dependent.
447 *Neurotoxicology*. 36, 112-7.
- 448 Hartz, A. M., et al., 2008. Diesel exhaust particles induce oxidative stress,
449 proinflammatory signaling, and P-glycoprotein up-regulation at the blood-brain
450 barrier. *FASEB J*. 22, 2723-33.
- 451 Hogg, J. C., van Eeden, S., 2009. Pulmonary and systemic response to atmospheric
452 pollution. *Respirology*. 14, 336-46.
- 453 Janssen, S., et al., 2008. Spatial interpolation of air pollution measurements using
454 CORINE land cover data. *Atmospheric Environment*. 42, 4884-4903.
- 455 Kicinski, M., et al., 2015. Neurobehavioral performance in adolescents is inversely
456 associated with traffic exposure. *Environ Int*. 75, 136-43.
- 457 Lauwerys, R., Hoet, P., 2001. Industrial chemical exposure: guidelines for biological
458 monitoring, third edition. CRC Press, Boca Raton.
- 459 Lefebvre, W., et al., 2013. Presentation and evaluation of an integrated model chain to
460 respond to traffic- and health-related policy questions. *Environmental Modelling &*
461 *Software*. 40, 160-170.

- 462 Letz, R., 2000. NES3 user's manual. Neurobehavioral Systems Inc., Atlanta (GA).
- 463 Maiheu, B., et al., 2012. Identifying the best available large-scale concentration maps for
464 air quality in Belgium. Available at:
465 [http://www.milieurapport.be/Upload/main/0_onderzoeksrapporten/2013/Eindrapport](http://www.milieurapport.be/Upload/main/0_onderzoeksrapporten/2013/Eindrapport_Concentratiekaarten_29_01_2013_TW.pdf)
466 [Concentratiekaarten_29_01_2013_TW.pdf](http://www.milieurapport.be/Upload/main/0_onderzoeksrapporten/2013/Eindrapport_Concentratiekaarten_29_01_2013_TW.pdf).
- 467 Oberdörster, G., et al., 2004. Translocation of inhaled ultrafine particles to the brain. *Inhal*
468 *Toxicol.* 16, 437-445.
- 469 Oberdorster, G., et al., 2002. Extrapulmonary translocation of ultrafine carbon particles
470 following whole-body inhalation exposure of rats. *J Toxicol Environ Health A.* 65,
471 1531-43.
- 472 Perera, F. P., et al., 2009. Prenatal airborne polycyclic aromatic hydrocarbon exposure and
473 child IQ at age 5 years. *Pediatrics.* 124, e195-e202.
- 474 Peterson, B. S., et al., 2015. Effects of prenatal exposure to air pollutants (polycyclic
475 aromatic hydrocarbons) on the development of brain white matter, cognition, and
476 behavior in later childhood. *JAMA Psychiatry.*
- 477 Proctor, S. P., et al., 2000. Validity of a computer-assisted neurobehavioral test battery in
478 toxicant encephalopathy. *Neurotoxicology.* 21, 703-14.
- 479 Saenen, N. D., et al., 2015. In utero fine particle air pollution and placental expression of
480 genes in the brain-derived neurotrophic factor signaling pathway: an ENVIRONAGE
481 birth cohort study. *Environ Health Perspect.* 123, 834-40.
- 482 Schroyen, C., et al., 2008. Internal exposure to pollutants measured in blood and urine of
483 Flemish adolescents in function of area of residence. *Chemosphere.* 71, 1317-1325.
- 484 Staessen, J. A., et al., 2001. Renal function, cytogenetic measurements, and sexual
485 development in adolescents in relation to environmental pollutants: a feasibility study
486 of biomarkers. *Lancet.* 357, 1660-1669.
- 487 Suglia, S. F., et al., 2008. Association of black carbon with cognition among children in a
488 prospective birth cohort study. *Am J Epidemiol.* 167, 280-286.
- 489 Suzuki, T., et al., 2010. In utero exposure to a low concentration of diesel exhaust affects
490 spontaneous locomotor activity and monoaminergic system in male mice. *Part Fibre*
491 *Toxicol.* 7, 7.
- 492 Tsukue, N., et al., 2009. Perinatal exposure to diesel exhaust affects gene expression in
493 mouse cerebrum. *Arch Toxicol.* 83, 985-1000.
- 494 van Berlo, D., et al., 2010. Comparative evaluation of the effects of short-term inhalation
495 exposure to diesel engine exhaust on rat lung and brain. *Arch Toxicol.* 84, 553-62.
- 496 Van Eeden, S. F., et al., 2001. Cytokines involved in the systemic inflammatory response
497 induced by exposure to particulate matter air pollutants PM10. *Am J Respir Crit Care*
498 *Med.* 164, 826-30.

- 499 VMM, 2012. Luchtkwaliteit in het Vlaamse Gewest. Jaarverslag immissiemeetnetten.
500 Kalenderjaar 2011. Vlaamse Milieumaatschappij.
- 501 Wang, S., et al., 2009. Association of traffic-related air pollution with children's
502 neurobehavioral functions in Quanzhou, China. *Environ Health Perspect.* 117, 1612-
503 1618.
- 504 White, R. F., et al., 2003. Neuropsychological screening for cognitive impairment using
505 computer-assisted tasks. *Assessment.* 10, 86-101.
- 506 Win-Shwe, T. T., et al., 2012. Nanoparticle-rich diesel exhaust affects hippocampal-
507 dependent spatial learning and NMDA receptor subunit expression in female mice.
508 *Nanotoxicology.* 6, 543-53.
- 509 Yokota, S., et al., 2009. Effect of prenatal exposure to diesel exhaust on dopaminergic
510 system in mice. *Neurosci Lett.* 449, 38-41.
- 511 Zanchi, A. C., et al., 2010. Pre and post-natal exposure to ambient level of air pollution
512 impairs memory of rats: the role of oxidative stress. *Inhal Toxicol.* 22, 910-8.
- 513 Zeltser, L. M., Leibel, R. L., 2011. Roles of the placenta in fetal brain development. *Proc*
514 *Natl Acad Sci U S A.* 108, 15667-8.
515