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DANKWOORD

SUMMARY

It is well-known that an exposure to toxic metals such as lead and methylmercury is harmful to the brain. A growing amount of evidence suggests that also other pollutants, including brominated flame retardants and traffic-related air pollution, may be neurotoxic. Children are particularly susceptible to the neurotoxic effects of environmental pollution due to the vulnerability of the developing brain and a higher exposure level of children compared to adults.

In this PhD project, we investigated the association between environmental pollution and neurobehavioral performance in the populations of primary school children and adolescents. We concentrated on three groups of pollutants: 1) brominated flame retardants, including polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), and tetrabromobisphenol A (TBBPA), 2) toxic metals, including lead, methylmercury, manganese, copper, arsenic, cadmium, thallium, and nickel, and 3) air pollution. The specific objectives of the project were as follows:

1) To investigate the association between exposure to brominated flame retardants and neurobehavioral performance in adolescents.
2) To investigate the association between exposure to toxic metals and neurobehavioral performance in adolescents.
3) To investigate the association between exposure to traffic-related air pollution and neurobehavioral performance in adolescents.
4) To investigate the association between acute changes in the air pollution level and neurobehavioral performance in primary school children.
5) To investigate the association between residential air pollution exposure and neurobehavioral performance in primary school children.

We conducted three epidemiological studies. In a cross-sectional study of 606 adolescents with a mean age of 14.9 years, we investigated the association between neurobehavioral performance and exposure to brominated flame retardants (Chapters 2), toxic metals (Chapter 3), and traffic (Chapter 4). Additional data on adolescents was gathered in a second cross-sectional study. We combined the data from these two studies and investigated using the data on 931 non-smoking adolescents the association between neurobehavioral performance and a specific indicator of traffic-related air pollution exposure, *trans*,*trans*-muconic acid, which is a urinary metabolite of benzene (Chapter 5). Finally, we investigated the association between air pollution exposure and neurobehavioral performance in a panel study of 334 primary school children with a mean age of 10.2 years (Chapter 6).

We measured neurobehavioral performance using the Neurobehavioral Evaluation System 3 (NES3), a computerized battery of neuropsychological tests. We used the following tests from the NES3 battery:

- the Continuous Performance Test, a test of sustained attention, which involves pressing a button when a target symbol appears on the screen;
- the Digit-Symbol Test, which assesses visual scanning and information processing speed;
- the Digit Span Test, a test of short-term memory, in which digits are reproduced after an oral presentation;
- the Pattern Comparison Test, a test of visuospatial information processing speed, in which the subject indicates which of the three patterns consisting of 100 blocks is different from the other two;
- the Finger Tapping Test, which assesses manual motor speed.

Besides the tests from the NES3 battery, we used a computerized version of the Stroop test to measure selective attention.

In line with experimental animal studies that observed effects of PBDEs on motor activity, exposure to PBDEs was associated with a decrease of manual motor speed in adolescents. This result supports the recent policies aiming to remove PBDEs from consumer products. We found no evidence of associations between PBDEs and sustained attention, short-term memory, or visual information processing speed. We also did not observe any consistent associations between neurobehavioral performance and HBCD or TBBPA (Chapter 2).

Blood copper was associated with sustained attention and short-term memory in adolescents with normal copper levels. However, the stratified analysis for gender revealed significant associations between copper and neurobehavioral performance only for girls. Our results add to the preliminary evidence of neurotoxic effects of a low-level copper exposure, suggesting the importance to further investigate these effects in future, preferably prospective, studies. The mean blood lead level was 13.8 µg/L. For this exposure level, no evidence of an association between the blood lead level and neurobehavioral performance was apparent. A low-level exposure to methylmercury, manganese, arsenic, cadmium, thallium, or nickel was not associated with neurobehavioral performance either (Chapter 3).

We found evidence of an association between traffic exposure and sustained attention in adolescents. In a study of 606 adolescents, we evaluated the exposure with a factor based on the urinary level of *trans*,*trans*-muconic acid and the amount of contact with traffic (using distance-weighted traffic density and time spent in traffic). A one standard deviation increase in this factor was associated with a 0.26 standard deviations decrease in sustained attention (95% Bayesian Credible Interval: -0.51 to -0.02, Chapter 4). A study based on an extended group including 931 non-smoking adolescents supported the existence of an association between traffic exposure and sustained attention. In this study, a ten-fold increase in the urine level of *trans*,*trans*-muconic acid was associated with a 0.17 standard deviations decrease in sustained attention (95% Confidence Interval: -0.31 to -0.04, Chapter 5).

In primary school children, we found no evidence of an association between day-to-day changes in the air pollution level and attention, short-term memory, or visual information processing speed. The average long-term residential particulate matter concentrations were associated with a decrease in attention. However, this association did not remain statistically significant in a sensitivity analysis, which took into account the school attended by the children. Additionally, the association was not statistically significant after correction for multiple testing related to the use of a number of different indicators of the long-term air pollution exposure. Since several recent studies reported an inverse association between a long-term exposure to traffic-related air pollution and neurobehavioral performance, our results may reflect that a long-term air pollution exposure is more relevant for neurobehavioral performance than day-to-day changes in the air pollution level (Chapter 6).

Observational human studies play an important role in human health risk assessment. The studies performed as a part of this PhD project contribute to the international research investigating the neurotoxic effects of environmental pollutants at the current exposure levels.

SAMENVATTING

Het is goed bekend dat een blootstelling aan lood en kwik schadelijk is voor de hersenen. Meer en meer studies suggereren dat ook andere polluenten zoals gebromeerde brandvertragers en luchtvervuiling neurotoxisch kunnen zijn. Kinderen zijn bijzonder vatbaar voor de neurotoxische effecten van milieuvervuiling omwille van de gevoeligheid van de hersenen tijdens de ontwikkelingsfase en omdat het blootstellingsniveau bij kinderen hoger ligt dan bij volwassenen.

In dit doctoraatsproject onderzochten we de associatie tussen milieuverontreiniging en neurocognitieve prestaties in de populaties van lagere schoolkinderen en adolescenten. We concentreerden ons op drie groepen van polluenten: 1) gebromeerde brandvertragers, waaronder PBDE's, HBCD en TBBPA, 2) toxische metalen, waaronder lood, kwik, mangaan, koper, arseen, cadmium, thallium en nikkel en 3) luchtvervuiling. De doelstellingen van het project waren de volgende:

1) de associatie tussen neurocognitieve vaardigheden en de blootstelling aan gebromeerde brandvertragers onderzoeken bij adolescenten,
2) de associatie tussen neurocognitieve vaardigheden en de blootstelling aan toxische metalen onderzoeken bij adolescenten,
3) de associatie tussen neurocognitieve vaardigheden en de blootstelling aan verkeersgerelateerde luchtvervuiling onderzoeken bij adolescenten,
4) de associatie tussen neurocognitieve vaardigheden en acute veranderingen van het niveau van luchtvervuiling onderzoeken bij lagere schoolkinderen,
5) de associatie tussen neurocognitieve vaardigheden en de residentiële blootstelling aan luchtvervuiling onderzoeken bij lagere schoolkinderen.

Er werden drie epidemiologische studies uitgevoerd. In een cross-sectionele studie van 606 adolescenten met een gemiddelde leeftijd van 14.9 jaar onderzochten we de associaties tussen neurocognitieve vaardigheden en de bloostelling aan gebromeerde brandvertragers (Hoofdstuk 2), toxische metalen (Hoofdstuk 3) en verkeer (Hoofdstuk 4). In een tweede cross-sectionele studie werden er bijkomende gegevens verzameld bij adolescenten. We voegden de data van deze twee studies samen en onderzochten de associatie tussen neurocognitieve vaardigheden en een specifieke indicator van de blootstelling aan verkeersgerelateerde luchtvervuiling, met name *trans*,*trans*-muconzuur (een metaboliet van benzeen in de urine, Hoofdstuk 5). Ten slotte onderzochten we de associatie tussen neurocognitieve vaardigheden en de blootstelling aan luchtvervuiling in een panelstudie van 334 lagere schoolkinderen met een gemiddelde leeftijd van 10.2 jaar (Hoofdstuk 6).

De neurocognitieve vaardigheden van de kinderen werden gemeten aan de hand van de batterij van computertesten Neurobehavioral Evaluation System

3 (NES3). We gebruikten de volgende testen van de NES3 batterij in onze studies:

- de Continuous Performance Test, een test van volgehouden aandacht waarbij gereageerd moet worden telkens wanneer er een welbepaald object op het computerscherm verschijnt;
- de Digit-Symbol Test die de snelheid waarmee visuele informatie verwerkt wordt, meet;
- de Digit Span Test, een test van het kortetermijngeheugen waarbij getallen moeten worden gereproduceerd;
- de Pattern Comparison Test die de snelheid waarmee visueel-ruimtelijke informatie verwerkt wordt, meet en waarbij de proefpersoon moet aanduiden welk van de drie patronen verschilt van de andere twee.
- de Finger Tapping Test, die de manuele motorische snelheid meet.

Daarnaast gebruikten we de Stroop Test om de selectieve aandacht te meten.

In lijn met de experimentele studies die effecten van PBDE's op de motorische activiteit van dieren waarnamen, was de blootstelling aan PBDE's bij de adolescenten geassocieerd met een lagere manuele motorische snelheid. We vonden geen aanwijzingen dat PBDE's geassocieerd waren met de volgehouden aandacht, het kortetermijngeheugen en de snelheid van het verwerken van visuele informatie. We observeerden geen associaties tussen neurocognitieve prestaties en HBCD of TBBPA (Hoofdstuk 2).

Koper in het bloed was geassocieerd met de volgehouden aandacht en het kortetermijngeheugen bij adolescenten met normale koperwaardes. In een analyse die voor geslacht gestratificeerd was, waren enkel de associaties bij meisjes statistisch significant. Onze resultaten dragen bij tot de preliminaire aanwijzingen dat koper, zelfs bij een lage blootstelling, neurotoxisch kan zijn. Dit dient bevestigt te worden in prospectief onderzoek. De gemiddelde concentratie van lood in het bloed was 13.8 µg/L. Voor dit blootstellingsniveau vonden we geen aanwijzingen van een associatie tussen lood en neurocognitieve prestaties. Ook bij kwik, mangaan, arseen, cadmium, thallium en nikkel werden er bij een laag blootstellingsniveau geen associaties gevonden met neurocognitieve vaardigheden van adolescenten (Hoofdstuk 3).

De blootstelling aan verkeer vertoonde bij adolescenten een negatieve associatie met de volgehouden aandacht. In een studie van 606 adolescenten werd het blootstellingsniveau bepaald aan de hand van de concentraties van *trans*,*trans*-muconzuur, de verkeersintensiteit en de hoeveelheid tijd die de proefpersonen doorbrachten in het verkeer. Wanneer de blootstelling aan verkeer met één standaarddeviatie steeg, daalde de volgehouden aandacht met 0.26 standaarddeviatie (95% Bayesiaanse betrouwbaarheidsinterval: -0.51 tot -0.02, Hoofdstuk 4). Dit resultaat werd bevestigd door een analyse waarin de steekproef uitgebreid werd tot 931 adolescenten. In deze analyse daalde de volgehouden aandacht met 0.17 standaarddeviatie (95%

betrouwbaarheidsinterval: -0.31 tot -0.04) wanneer de concentratie van *trans*,*trans*-muconzuur in de urine tien keer verhoogde (Hoofdstuk 5).

Bij lagere schoolkinderen waren acute veranderingen in het niveau van luchtvervuiling niet geassocieerd met de aandacht, het kortetermijngeheugen en de snelheid van het verwerken van visuele informatie. We vonden aanwijzingen van een negatieve associatie tussen de residentiële lange-termijnblootstelling aan fijn stof en de aandacht. In een sensitiviteitsanalyse waarin gecorrigeerd werd voor de school waar de kinderen naartoe gingen, was deze associatie niet meer statistisch significant. Bovendien was deze associatie ook niet statistisch significant wanneer rekening werd gehouden met het feit dat er meerdere blootstellingsmerkers gebruikt werden in de analyse (multiple testing). Een aantal recente studies hebben een negatief verband gerapporteerd tussen een lange-termijnblootstelling aan luchtvervuiling en cognitieve vaardigheden van kinderen. In combinatie met onze resultaten zou dit erop kunnen wijzen dat een lange-termijnblootstelling aan luchtvervuiling relevanter is voor de cognitieve functie dan temporele variatie in de luchtkwaliteit (Hoofdstuk 6).

Epidemiologische studies spelen een belangrijke rol bij het evalueren van de gezondheidsrisico's voor mensen. De studies die uitgevoerd werden in het kader van dit doctoraatsproject leveren een bijdrage aan het internationale onderzoek rond de neurotoxische effecten van milieuvervuiling.

LIST OF ABBREVIATIONS

ADHD	attention deficit hyperactivity disorder
BC	black carbon
BCI	Bayesian credible interval
BFR	brominated flame retardant
BMI	body mass index
CI	confidence interval
DWTD	distance-weighted traffic density
EC	elemental carbon
HBCD	hexabromocyclododecane
IFDM	Immission Frequency Distribution Model
IQ	intelligence quotient
IQR	interquartile range
LOD	limit of detection
LOQ	limit of quantification
NES	Neurobehavioral Evaluation System
NES3	Neurobehavioral Evaluation System, version 3
PAH	polycyclic aromatic hydrocarbon
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PM	particulate matter
PM_{10}	particulate matter with an aerodynamic diameter of less than 10 µm
$PM_{2.5}$	particulate matter with an aerodynamic diameter of less than 2.5 µm
SD	standard deviation
t,t-MA	*trans*,*trans*-muconic acid
t,t-MA-U	*trans,trans*-muconic acid in urine
TBBPA	tetrabromobisphenol A
TLV	threshold limit value
UFP	ultrafine particle
WHO	World Health Organization

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Chapter 1

General introduction*

*Partly based on: Kicinski M, Nawrot T. Neurobehavioral effects of air pollution in children. In: *Environmental factors in neurodevelopmental and neurodegenerative disorders*. Edited by: Costa L, Aschner M; Elsevier; 2015.

According to the European Community Inventory, more than 100,000 chemical substances were present in Europe in 2008 [1]. The global chemical industry continues to expand [2] and each year thousands of new substances enter the market [1]. The large scale use of chemicals causes a widespread contamination of the environment. Pollutants are present in the air, water, soil, plants, animals, food products, and human tissues. A Flemish study measured the concentrations of 41 biomarkers of exposure to chemical substances including toxic metals, chlorinated hydrocarbons, brominated flame retardants, phthalates, organophosphate pesticides, benzene, and other chemicals in blood, urine, or hair samples of adolescents [3]. Out of these biomarkers, 26 were found in the samples of more than 90% of the study participants. Since a large number of individuals is exposed to environmental pollution, environmental risk factors have important public health consequences [4].

In the twentieth century, a number of environmental disasters took place [5]. The London's lethal smog in 1952 with an estimated death toll of 12,000 people is a well-known example [6]. In response to the disaster in England, the Parliament of the United Kingdom passed in 1956 the Clean Air Act, which was one of the precursors of environmental regulations. Today, environmental laws exist in all developed countries of the world. Environmental research plays an important role in the process of creating new and updating the existing regulations by providing data on the health effects of pollutants for certain exposure levels.

The central nervous system is particularly vulnerable to toxic insults [7]. As a result of the technological development and the environmental regulations, exposures to pollutants at levels causing strong acute neurotoxicity are nowadays uncommon in the developed countries. However, exposures at the current levels may still cause subtle neurobehavioral effects. One reason why it is important to investigate whether they do is that such subtle neurobehavioral deficits may, with prolonged exposure, progress to more severe effects [8-10].

Due to their unique vulnerability, children are a population of special interest in studies of neurotoxic effects of environmental pollution [11-14]. For many environmental pollutants the level of exposure of children is higher compared to adults. For instance, children inhale more pollutants from the air because they spend more time outdoors and have a higher breathing rate [15]. Children's physiologic needs for more water and food per kilogram of body weight compared with adults are other reasons of an increased exposure to environmental pollutants. Moreover, exposure to toxicants at a certain level may affect children to a greater degree than adults [13]. The nervous system of children undergoes developmental processes. A disturbance of these processes may have a large impact on the nervous system [13,14]. The neurotoxic effects of a low-level lead exposure in children exemplify a particular vulnerability of this age group to neurotoxic insults [12].

BROMINATED FLAME RETARDANTS

Brominated flame retardants (BFRs) are chemicals used in electrical appliances such as TV sets, computers, mobile phones, and kitchen appliances, textiles, building materials, and plastics to prevent fire [16,17]. Widely used brominated flame retardants include polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), and tetrabromobisphenol A (TBBPA). The large scale production of BFRs started in the 1970s and gradually increased until the end of the twentieth century, reaching 216,800 tons in 2000 [18].

Humans are exposed to BFRs via contaminated food, indoor dust, and other pathways [17,19]. Indeed, the use of BFRs led to an accumulation of these chemicals in human tissues [17,19], as exemplified by a Swedish study of the concentrations of PBDEs in breast milk [20] (Figure 1). In the beginning of the 21st century, the use of PBDEs decreased due to environmental regulations banning them and voluntary actions of the industry [17,21]. However, these chemicals are expected to remain in the environment for many years due to their persistency and the presence in products manufactured before the introduction of the policies aiming to eliminate them [17,21]. Other brominated flame retardants including HBCD and TBBPA are still in use.

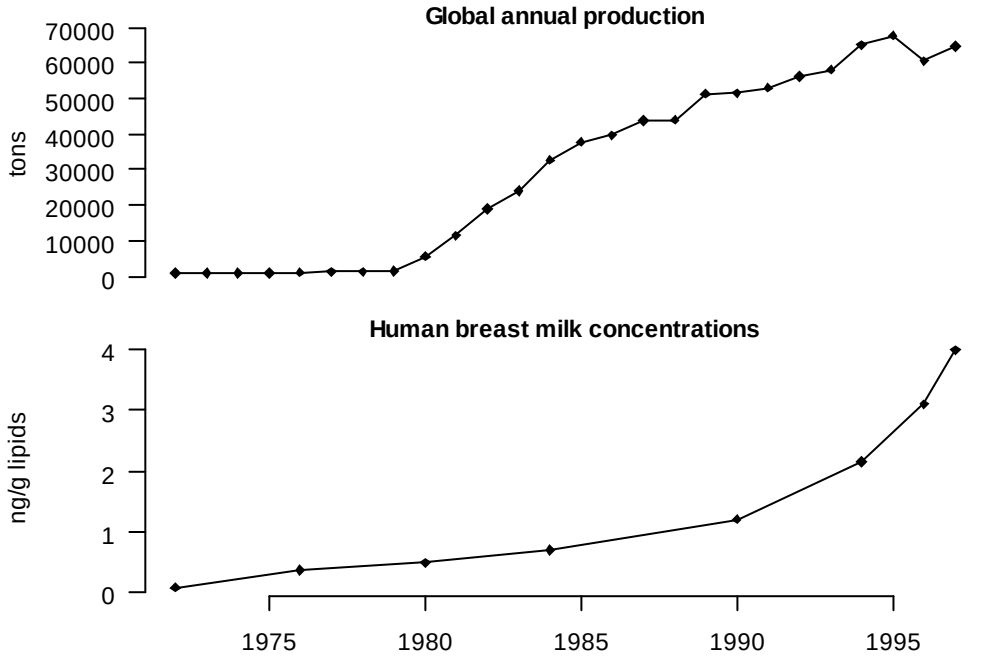

FIGURE 1. Estimates of the global annual PBDE production [18] and human breast milk concentrations of PBDEs in Sweden from 1972 to 1997 [20].

A number of animal studies showed that BFRs affect neurodevelopment [19,22,23]. Changes in the motor activity have been most frequently studied and best-documented [19,23]. However, human studies are scarce. In 2011, when this PhD project started, little was known about the neurobehavioral effects of BFRs in humans. To my best knowledge, only three human studies investigating the association between exposure to BFRs and neurobehavioral performance existed at that time [24-26]. One of these three studies reported an association between the concentrations of several PBDE congeners in umbilical cord blood and the level of mental development at the age of two and intelligence at the age of two and three [25], providing preliminary evidence of neurobehavioral effects of BFRs in humans. The results of the other two studies were inconsistent. All three studies were small (included less than 150 participants in the analyses). Clearly, the neurobehavioral effects of BFRs in humans needed further investigation.

TOXIC METALS

Lead

The word *plumber* has its origins in the Latin word *plumbum*, which means lead, and the widespread presence of lead in the environment dates back to the Roman times when the metal was used to make water pipes [27]. Starting not later than in the Roman times, lead additives were used to preserve and sweeten wine [28].

The neurotoxic properties of lead were already known in the ancient times [29,30]. Dioscorides, a Greek physician, noted in the first century AD that "lead makes the mind give away" [29]. Colica pictonum, a disease of the central nervous system, was widespread from the Roman times until the eighteenth century [28]. The symptoms of colica pictonum included headache, fatigue, fever, restlessness, insomnia, loss of appetite, lethargy, colic pains, loss of speech, deafness, blindness, and paralysis. In some cases, the disease was lethal. At the end of the seventeenth century, the German physician Eberhard Gockel discovered that lead was the cause of colica pictonum by linking the epidemic of the disease to drinking wine with large amounts of lead additives [28].

Despite the knowledge of its neurotoxic properties, lead continued to be widely used in the twentieth century. As a result, the cases of lead poisoning of children were still common in the middle of the previous century [29,31]. During the latter part of the twentieth century, reports describing the association between a chronic low-level lead exposure and the neurobehavioral function

became to appear [29,32]. Most remarkably, a number of epidemiological studies reported a negative association between blood lead level and intelligence of children [33]. As a result of the phase-out of lead-based paint and the removal of lead from gasoline that started in the 1970s, the level of lead exposure substantially decreased in the USA and Europe.

Nowadays, blood lead levels above 10 µg/dL, which used to be regarded as the safety threshold for children exposure, are uncommon in the Western countries [34,35]. However, recent studies show that lead exposure continues to be associated with neurobehavioral performance for blood lead levels below 10 µg/dL [36-39]. In fact, even studies with the mean blood lead concentrations below 2 µg/dL reported associations between lead exposure and neurobehavioral outcomes in children [40,41]. Therefore, it is unclear whether the current lead exposure can be assumed safe.

Methylmercury

Similar to lead, mercury has been used since antiquity [42]. In the 1950s, an environmental disaster in Japan called the attention to the neurotoxic effects of methylmercury [43]. A deposition of mercurial waste in the Minamata Bay harshly affected the local population with a fish-rich diet. The symptoms of methylmercury poisoning included visual impairment, hearing impairment, changes in the sense of smell, loss of taste, disturbances of complex movements, alterations of speech, and tremor. In the beginning of the 1970s, the epidemic of the disease in Iraq caused by consumption of bread prepared from seed wheat treated with a methylmercurial fungicide reminded the world about the neurotoxic effects of the chemical [44].

Cohorts from the Faroe Islands and the Seychelles provide information about the association between neurobehavioral performance and methylmercury exposure resulting from extensive consumption of fish and other seafood from the ocean. In children from the Faroe Islands, a prenatal methylmercury exposure was associated with cognitive and motor changes [45]. However, methylmercury exposure was not associated with neurobehavioral outcomes in children from the Seychelles exposed to methylmercury to a similar extent as those from the Faroe Islands [46]. Results of studies with lower exposure levels are inconsistent as well [47-50].

Essential elements manganese and copper

The essential elements manganese and copper are neurotoxic at high doses. Our knowledge about the neurological effects of manganese is largely based on occupational studies that observed motor and cognitive deficits in exposed workers [51-53]. Dramatic consequences of an excessive copper exposure are apparent in the clinical context. Wilson's disease, an autosomal

recessive disorder resulting in an accumulation of copper in tissues including the brain, causes severe neurological symptoms [54]. As both deficiency and excess of manganese and copper can be harmful, the optimal levels need to be carefully determined. This is difficult at this moment due to the scarcity of data. Preliminary evidence suggests that both environmental manganese [55,56] and copper [57-59] exposure may be neurotoxic at the current exposure levels.

Other toxic metals

Arsenic, a potent toxicant, can also damage the central nervous system [60]. In children highly exposed through contaminated ground water, cognitive performance was negatively associated with the urine levels of this metal [61-63]. The neurobehavioral effects of a low-level exposure to arsenic remain largely unexplored. Some [57,64,65] but not all [66,67] studies on the neurobehavioral effects of cadmium exposure revealed a negative association. Human cases of poisoning and animal studies have showed that nickel [68,69] and thallium [70,71] are neurotoxic at high exposure levels. However, little is known about the neurobehavioral effects of these heavy metals at a low level of exposure.

AMBIENT AIR POLLUTION

Composition

The exact composition of air pollution depends on the sources of emissions. In Western countries, where vehicle traffic is a major contributor to ambient air pollution, the most important air contaminants include toxic metals, nitrogen oxides, such as NO_2 and NO, ozone, black carbon, inorganic carbon compounds such as CO, and organic compounds such as polycyclic aromatic hydrocarbons and benzene.

Particulate matter (PM) is a mixture of small particles and liquid droplets in the air. Particles with an aerodynamic diameter of less than 10 μm (PM_{10}) are of special interest because they can enter the lungs. PM_{10} includes coarse particles, which have an aerodynamic diameter between 2.5 μm and 10 μm, and fine particles, which have an aerodynamic diameter of less than 2.5 μm ($PM_{2.5}$). Coarse particles are largely constituted by salt, nitrate, and road dust elements, such as calcium and manganese [72,73]. $PM_{2.5}$ includes sulfate, nitrate, organic compounds, black carbon, and toxic metals such as lead [72,73]. Ultrafine particles (UFPs) are particles with a diameter of less than 0.1 μm. Due to their small size, UFPs are deposited deep in the lungs, translocate to the systemic circulation, and can even reach the brain [74].

Health effects of ambient air pollution

Air pollution is a recognized risk factor for cardiovascular and respiratory diseases. Daily increases in the level of air pollution are related to a higher risk of respiratory symptoms and cardiovascular events including angina, myocardial infarction, heart failure, and death [75-79]. Studies of the long-term effects of air pollution exposure observed that living in areas with higher levels of air pollution is associated with a slower development of the lung function in children, a higher risk of cardiopulmonary diseases, and an increased mortality [79-81]. According to the Global Burden of Disease Study of the World Bank and the World Health Organization, ambient air pollution exposure is the ninth most important health risk factor worldwide, with an estimated global burden of 76.1 million healthy life-years per year [82].

Air pollution as a neurotoxicant

There is a substantial amount of evidence showing that exposure to traffic-related air pollution induces neurotoxicity in the brain. The effects observed in experimental studies in rodents include changes in gene expression [83-85] and the level and turnover of neurotransmitters [86-91], oxidative stress [91-93], pro-inflammatory cytokine response [85,86,94-100], glial activation [99,101], and neuron apoptosis [102,103]. These animal studies indicate the need to examine the hypothesis of neurobehavioral effects of traffic-related air pollution in humans.

A study based on a sample of 55 children observed a poorer performance in cognitive tasks of children living in New Mexico compared to those living in a clean city, and linked this observation to the presence of prefrontal white matter hyperintense lesions [104]. A study of 202 children from Boston found an association between intelligence and the average lifetime residential exposure to black carbon, a marker for traffic-related air pollution [105]. A Chinese study, which administered a battery of neurobehavioral tests in 928 nine years old children attending two schools with different concentrations of ambient PM_{10} and NO_2, reported that those from a polluted area performed weaker in cognitive tasks [106]. Another study assessed the exposure to airborne polycyclic aromatic hydrocarbons (PAHs) during the third trimester of pregnancy in a cohort from New York using personal air monitors [107]. A higher exposure was associated with a slower mental development at age three [108] and a lower intelligence at age five [109]. A similar Polish study supported the existence of an association between prenatal PAHs exposure and intelligence of children [110]. In contrast, residential exposure to traffic-related air pollution indicated by NO_2 concentrations was not associated with cognitive functions in a cross-sectional Spanish study of 210 four years old children [111].

The preceding paragraph describes the epidemiological studies investigating the association between traffic-related air pollution and

neurobehavioral outcomes in children that existed in January 2011, when the proposal of this PhD project was submitted. Most of these studies were relatively small and provided preliminary evidence of an association between air pollution and the neurobehavioral function. None of them considered the effects of a short-term exposure.

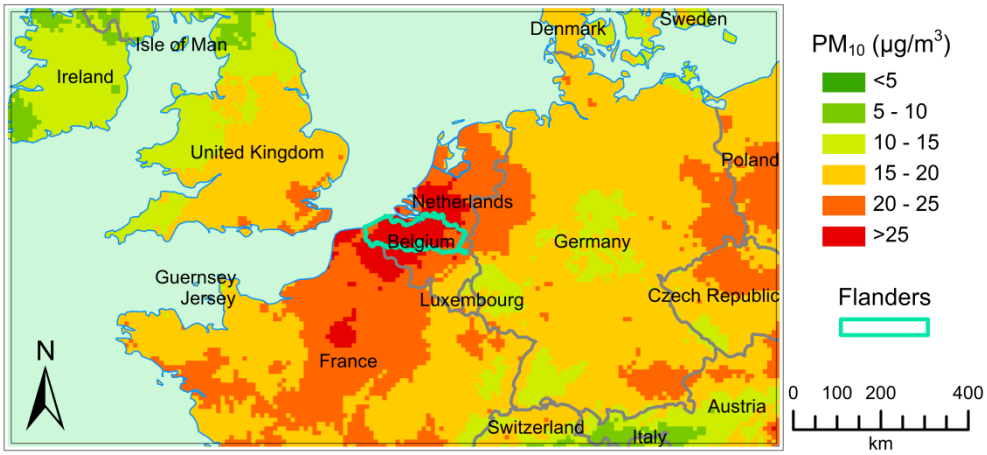

FIGURE 2. The average annual PM_{10} concentrations in 2009 in Flanders and its surroundings.

Data source: the European Topic Center on Air Pollution and Climate Change Mitigation [112].

Flanders: an air pollution hotspot area

Flanders, a region in the north of Belgium with approximately 6 million inhabitants, is characterized by a high population density and the presence of the urban sprawl requiring the inhabitants to travel frequently and relatively far [113]. Cars are the most popular means of transport of the Flemish people [113]. Trucks using the Flemish roads due to the central location of the region in Europe contribute further to traffic emissions. The total number of vehicles in Flanders exceeded 3.7 million in 2009 [114]. It has been estimated that in the same year, these vehicles travelled 56.4 billion km [114].

One consequence of the high population density and the high traffic density is a high level of air pollution at locations inhabited by people. Indeed, the north of Belgium is one of the European air pollution hotspot areas (Figure 2) [112]. In 2009, the estimates of the annual mean concentrations of PM_{10} significantly exceeded the World Health Organization guideline of 20 $\mu g/m^3$ in Flanders (Figure 2) [112]. Additionally, peaks of air pollution are common. In 2009, the World Health Organization guideline of a maximum of three days of daily average concentrations above 50 $\mu g/m^3$ was exceeded for all (both rural and urban) measuring stations in Flanders [115]. Epidemiological studies showed that air pollution in Flanders is associated with cardiovascular [116-118]

and respiratory health outcomes [119,120] and an increased mortality [121,122].

NEUROBEHAVIORAL TESTS

Historical note

By the middle of the twentieth century, our knowledge about the neurological effects of chemicals was largely based on easily observable pathology (e.g., tremors) resulting from high-level exposures [123]. Due to environmental regulations and technological developments cases of easily observable neurotoxicity of environmental pollutants became less common in the Western countries in the second half of the twentieth century. Consequently, the interest of neurotoxicology shifted to more subtle effects.

The advancements of psychometrics provided tools allowing to study such subtle effects. In the beginning of the twentieth century, the first standardized tests measuring specific neurobehavioral abilities and intelligence were developed, providing a way to quantitatively evaluate neurobehavioral performance [124]. By 1960, the first versions of many of neurobehavioral tests that are widely used nowadays already existed, including the Continuous Performance Test [125], the Digit Symbol Test [126], the Digit Span Test [127], and the Stroop Test [128]. In the seventies and eighties of the twentieth century, neurobehavioral tests proved to be a sensitive measure of toxicity of the central nervous system in occupational studies [129,130]. Nowadays, neurobehavioral testing is a standard approach to investigate the neurotoxic effects of environmental pollution [123].

Neurobehavioral Evaluation System (NES)

The Neurobehavioral Evaluation System (NES) is a battery of commonly used neurobehavioral tests that was developed to study the neurological effects of exposures to environmental agents [131]. It is a computerized system. The advantages of this approach include efficiency of data collection and a rigid standardization of the testing protocol through a computer program. NES is one of the most frequently used testing systems in epidemiological studies [123].

The Neurobehavioral Evaluation System 3 (NES3) is the third version of the NES battery [132,133]. NES3 is administered on a computer equipped with a touch screen (Figure 3). All responses are made by touching the screen. The test instructions are given verbally by the computer, making the testing procedure completely standardized. A feasibility study of a Dutch adaptation of NES

showed that the battery is appropriate for use in eight years old or older children [134].

There are 17 tests available in NES3, including:

- the Continuous Performance Test, a test of sustained attention, which involves pressing a button when a target symbol appears on the screen;
- the Digit-Symbol Test, which assesses visual scanning and information processing speed;
- the Digit Span Test, a test of short-term memory, in which digits are reproduced after an oral presentation in the order of the presentation (Digit Span Forward) and in the reverse order (Digit Span Backward);
- the Pattern Comparison Test, a test of visuospatial information processing speed, in which a subject indicates which of the three patterns consisting of 100 blocks is different from the other two;
- the Finger Tapping Test, which assesses manual motor speed.

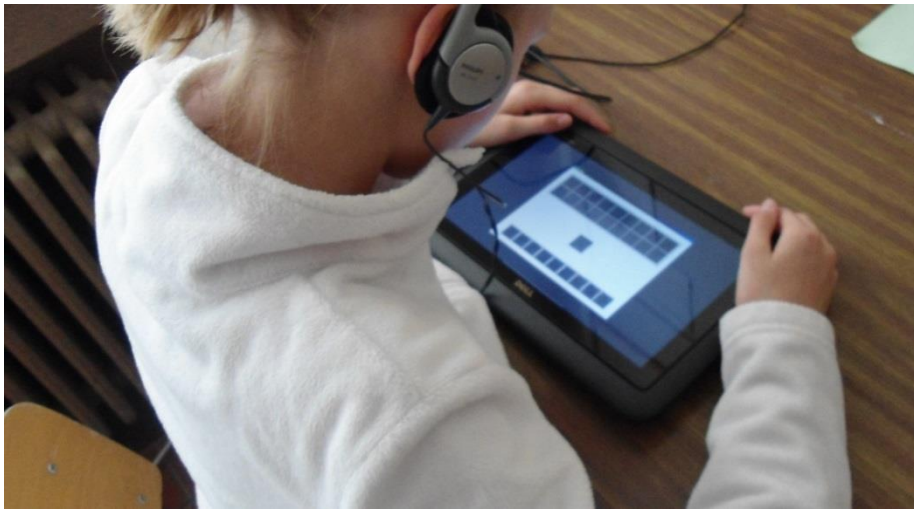

FIGURE 3. A child working on a NES3 test on a touch screen computer.

OBJECTIVES

In this PhD project, we investigated the association between environmental pollution and neurobehavioral performance in the populations of primary school children and adolescents. We used the NES3 battery to measure neurobehavioral performance. We concentrated on the effects of three groups of pollutants described in the preceding sections, that is: brominated flame-

retardants, toxic metals, and air pollution. The specific objectives were as follows:

1) To investigate the association between exposure to brominated flame retardants and neurobehavioral performance in adolescents (Chapter 2).
2) To investigate the association between exposure to toxic metals and neurobehavioral performance in adolescents (Chapter 3).
3) To investigate the association between exposure to traffic-related air pollution and neurobehavioral performance in adolescents (Chapter 4 and Chapter 5).
4) To investigate the association between acute changes in the air pollution level and neurobehavioral performance in primary school children (Chapter 6).
5) To investigate the association between residential air pollution exposure and neurobehavioral performance in primary school children (Chapter 6).

In order to reach these objectives, we conducted three epidemiological studies. In a cross-sectional study of adolescents, we investigated the association between environmental pollution and neurobehavioral performance (Chapters 2-4). Additional data on adolescents was gathered in a second cross-sectional study. We combined the data from these two studies in order to investigate the association between a specific indicator of traffic-related air pollution exposure, a urinary metabolite of benzene *trans*,*trans*-muconic acid, and neurobehavioral performance (Chapter 5). The association between air pollution exposure and neurobehavioral performance in primary school children was investigated in a panel study (Chapter 6).

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Chapter 2

Neurobehavioral function and low-level exposure to brominated flame retardants in adolescents: a cross-sectional study*

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ABSTRACT

Introduction: Animal and in vitro studies demonstrated a neurotoxic potential of brominated flame retardants, a group of chemicals used in many household and commercial products to prevent fire. Although the first reports of detrimental neurobehavioral effects in rodents appeared more than ten years ago, human data are sparse.

Methods: As a part of a biomonitoring program for environmental health surveillance in Flanders, Belgium, we assessed the neurobehavioral function with the Neurobehavioral Evaluation System (NES3), and collected blood samples in a group of high school students. Cross-sectional data on 515 adolescents (13.6-17 years of age) was available for the analysis. Multiple regression models accounting for potential confounders were used to investigate the associations between biomarkers of internal exposure to brominated flame retardants [serum levels of polybrominated diphenyl ether (PBDE) congeners 47, 99, 100, 153, and 209, hexabromocyclododecane (HBCD), and tetrabromobisphenol A (TBBPA)] and cognitive performance. In addition, we investigated the association between brominated flame retardants and serum levels of FT3, FT4, and TSH.

Results: A two-fold increase of the sum of serum PBDEs was associated with a decrease of the number of taps with the preferred-hand in the Finger Tapping Test by 5.31 (95% CI: 0.56 to 10.05, p=0.029). The effects of the individual PBDE congeners on the motor speed were consistent. Serum levels above the limit of quantification were associated with an average decrease of FT3 level by 0.18 pg/mL (95% CI: 0.03 to 0.34, p=0.020) for PBDE-99 and by 0.15 pg/mL (95% CI: 0.004 to 0.29, p=0.045) for PBDE-100, compared with concentrations below the limit of quantification. PBDE-47 level above the limit of quantification was associated with an average increase of TSH levels by 10.1% (95% CI: 0.8% to 20.2%, p=0.033), compared with concentrations below the limit of quantification. We did not observe effects of PBDEs on neurobehavioral domains other than the motor function. HBCD and TBBPA did not show consistent associations with performance in the neurobehavioral tests.

Conclusions: This study is one of few studies and so far the largest one investigating the neurobehavioral effects of brominated flame retardants in humans. Consistently with experimental animal data, PBDE exposure was associated with changes in the motor function and the serum levels of the thyroid hormones.

INTRODUCTION

Brominated flame retardants (BFRs) are chemicals widely used in a variety of household and commercial products including plastics, electric equipment, textiles, and polyesters in order to prevent fire [1,2]. Many of them bioaccumulate in the environment and have been found in water, air, biota, human tissues, breast milk, and blood [3-6]. House dust and food represent two important sources of human exposure [5,7].

A number of animal studies showed effects of a prenatal and postnatal exposure to BFRs on neurodevelopment and were recently reviewed [8-10]. Neurobehavioral effects during juvenile development or adulthood have been observed in rodents after a brief postnatal exposure to polybrominated diphenyl ethers (PBDE) 47 [11,12], 99 [11,13-16], 153 [17], 203 [18], 206 [18], 209 [19-22], the commercial PBDE mixture DE-71 [23,24], and hexabromocyclododecane (HBCD) [25,26], a chronic perinatal exposure to PBDE-47 [27,28] and PBDE-99 [29], and an acute prenatal exposure to PBDE-99 [30]. Detrimental effects of PBDE exposure on neurodevelopment have also been reported in zebrafish [31,32]. Changes in the motor activity have been most frequently studied and best documented [8,10]. Also in vitro studies support the hypothesis of neurotoxicity of BFRs. PBDE congeners were capable of inducing oxidative stress [33-35] and apoptosis [33,34] in cultured neurons.

Despite the fact that the first results of the experimental animal studies suggesting a neurotoxic potential of BFRs were available more than 10 years ago, the effects in humans have not been extensively investigated to date. Three small prospective studies [36-38] evaluated the effects of a perinatal exposure to BFRs on neurobehavioral function in children. Concentrations of several PBDE congeners in umbilical cord blood of newborns showed an association with indicators of neurodevelopment in early childhood. [37] In another study [36], consistent neurodevelopmental effects at the age of 8-12 months of the exposure measured by breast milk PBDE concentrations were not observed. Roze et al. [38] reported both negative and positive neurobehavioral effects of a prenatal exposure to HBCD and several PBDE congeners among 5-6 years old children. In a cross-sectional study on older adults [39], PBDEs measured in the serum were not associated with performance in cognitive tasks. All associations reported in these studies were investigated using less than 150 participants.

The thyroid system is one of the targets of BFRs [40]. Experimental animal studies have demonstrated that a PBDE exposure may result in a decrease of blood thyroxine [22,24,41-45] and triiodothyronine [43,44,46-48] levels. These effects were observed not only in gestation and early childhood, but also later in life [41,42]. A disruption of the thyroid system has been suggested as a mediator of the BFRs neurotoxicity [9,49].

We conducted a cross-sectional study of the association between neurobehavioral function and biomarkers of exposure to BFRs [serum levels of polybrominated diphenyl ether (PBDE) congeners 47, 99, 100, 153, and 209, hexabromocyclododecane (HBCD), and tetrabromobisphenol A (TBBPA)] in a group of Flemish adolescents. Additionally, we investigated the association between BFRs and the thyroid function as a potential biological mechanism responsible for the neurotoxicity of these chemicals.

METHODS

Study population and data collection

The study was a part of a biomonitoring program for environmental health surveillance in Flanders, Belgium. The participants were recruited between 2008 and 2011 in two industrial areas (Genk and Menen) and from the general population of Flemish adolescents. The participants were eligible if they studied in the third year of a secondary school. Hence, most participants were 14 or 15 years old, but older students were also allowed in the study. Only adolescents who spoke Dutch were eligible.

In the general Flemish population, random sampling was attained through a multistage sampling design. In the first step, ten schools (two in each of the five Flemish provinces) - at least 20 km apart from each other - were randomly selected. In the second step, classes were randomly selected within each school and all pupils in a class were invited until the provided number of participants was reached. The number of participants per province was proportional to the number of inhabitants in that province (status at 01/01/2006).

In Genk and Menen, study areas were defined based on environmental data and the location of the industrial sites. Names and addresses were attained from the population registry. In Genk, 54% of the adolescents were invited via a letter sent to the home address and 46% during a home visit. In Menen, all participants were invited via a letter sent to the home address. Due to a small response, 30% of the invited children were contacted again via schools and 9% via a home visit.

Two weeks before the study session, subjects received two questionnaires to fill in, one for themselves and one for their parents. The questionnaire for adolescents included information about their exercising habits, amount of time spent using a computer, alcohol use, and smoking. Questions about the socioeconomic status, passive smoking and eating habits were included in the questionnaire for parents. The study session including an

administration of the neurological tests, a collection of a blood sample, and a measurement of the length and the weight was approximately one hour long. Each subject received a 10 Euro voucher for the participation. Both parents and teenagers provided informed consent for participation. The study was approved by the Ethical Committee of the University of Antwerp.

The response rate equaled 22.1% in the general Flemish population, 34.3% in Genk and 22.5% in Menen. A non-responder analysis performed in a group of 106 adolescents (30 participants and 76 non-participants) did not reveal differences in the socio-economic status indicators type of education (general secondary education versus other, p=0.58), education of the father (higher education vs not, p=0.99), or education of the mother (higher education vs not, p=0.22) between participants and non-participants. The proportion of girls was higher among the participants (83.3 % vs. 61.8% in non-participants, p=0.03), but an equal distribution between boys and girls was a stratification criterion in the recruitment strategy. 606 adolescents participated in the study. Blood measurements were not available for three participants, and four participants did not complete any of the neurobehavioral tests. Additionally, for 84 out of the remaining 599 participants information on at least one of the covariates used in the analysis was missing. 515 subjects who completed at least one neurobehavioral test and for whom information about the covariates and serum BFRs levels was available, were used in the analysis (see Table 1). This group consisted of 163 adolescents from Genk, 178 from Menen, and 174 from the general Flemish population.

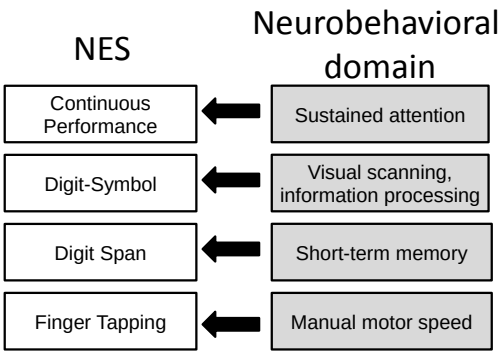

FIGURE 1. An overview of the neurological tests used in the study.

Neurobehavioral tests

Neurobehavioral Evaluation System (NES) is a computerized battery of tests developed to study the neurological effects of an exposure to environmental agents [50]. NES has been used in a number of studies investigating the neurobehavioral impact of neurotoxicants and dose-response

relationships with intensity of exposure were reported [51]. In our study, we used four tests from the NES3 version of the test battery [52] (see Figure 1).

In the Continuous Performance Test, a series of letters is displayed on the screen, one at a time, and each for approximately 200msec. The task is to immediately respond to the letter S, and not to other letters, by pressing the spacebar. A new letter appears each 1000msec. In total, the letter S appears 60 times. The performance indicators are calculated based on the last 48 appearances. The mean reaction time for responding to the target letter in msec, the number of errors of omission, i.e., the number of times that a subject did not react within 1200msec, the number of errors of commission, i.e., the number of responses when the letter S was not displayed, were used as the measure of performance. The test evaluates sustained attention. It showed a good test-retest reliability in a group of patients directed to a neuropsychological examination [51].

In the Digit-Symbol Test, a row of 9 symbols paired with 9 digits is displayed at the top of the screen. The same 9 symbols but in a different order are shown at the bottom. When a digit is displayed, the task is to indicate the symbol, which is paired with this digit, from the bottom row. A new digit appears only after the correct symbol has been indicated. In total, 27 digits are displayed. The total time needed to complete the test measured in seconds describes the performance. The test is characterized by a satisfactory reliability [52]. In a part of the study area, a different test was administered instead of the Digit-Symbol Test. As a result, the results of this test were only available for a group of 341 participants from Genk and Menen.

The Digit Span Test consists of two parts. In the first part, a subject hears a sequence of digits. The task is to reproduce them. In case of a correct answer, a one digit longer sequence is presented. In case of a mistake, a sequence of the same length is presented. When two incorrect answers in a row are given, the first part of the test finishes. The second part is the same as the first one, but the sequences are reproduced in the reverse order. Digit Span Forward is the maximum number of digits reproduced in the first part. Digit Span Backward is the maximum number of digits reproduced in the reverse order. The first part of the test assesses the working memory span. Good performance in the second part requires both the ability to hold and manipulate information. In a part of the study area, the Digit Span Test was administered using computers with touch screens. In this case the task was to indicate the digits on the screen, not using the keyboard.

In the Finger Tapping Test, a subject presses the spacebar as many times as possible during a trial of 10 sec. The first part of the test consists of 4 trials with the preferred-hand. The second part consists of 4 trials with the non-preferred hand. The summary measures are the total number taps with the preferred-hand and the total number of taps with the non-preferred hand. The test measures manual motor speed.

TABLE 1. Descriptive statistics.

N=515[a]	
Anthropometric measurements	
Boys	271 (52.6%)
Age, years	14.9 (0.7)
BMI, kg/m^2	20.4 (3.1)
Lifestyle	
Type of education – general secondary	290 (56.3%)
The highest level of education of parents	
no diploma	22 (4.3%)
9 grades	52 (10.1%)
12 grades	163 (31.7%)
College of university diploma	278 (54%)
Parents owning the house	456 (88.5%)
Current smoking	65 (12.6%)
Passive smoking[b]	82 (15.9%)
Alcohol use at least once a month	129 (25%)
Number of hours a week using computer	
< 2	50 (9.7%)
2-9	271 (52.6%)
10-19	147 (28.5%)
≥ 20	47 (9.1%)
Fish consumption[c]	
Low	224 (43.5%)
Average	186 (36.1%)
High	105 (20.4%)
Physical activity in leisure time at least once a week	405 (78.6%)
Biochemical measures	
Blood lipids, mg/dl	448.9 (72.9)
Blood lead, µg/dl	1.4 (0.7 to 2.9)
Sum of serum PCB 138, 153, and 180, ng/L	171.6 (58 to 445)
Thyroid hormones serum levels	
FT3, pg/mL	4.15 (0.53)
FT4, ng/dL	1.24 (0.17)
TSH, µU/mL	2.15 (0.99 to 4.45)
Neurobehavioral parameters	
Continuous Performance, reaction time, msec, N=489	409.2 (41.8)
Continuous Performance, errors of omission, N=489	2.3 (2.7)
Continuous Performance, errors of commission, N=489	5.6 (3.5)
Digit-Symbol, sec, N=340	98.3 (17.7)
Digit Span Forward, N=511	5.6 (1.03)
Digit Span Backward, N=499	4.49 (1.01)
Finger Tapping, preferred hand, N=511	293.7 (40.2)
Finger Tapping, non-preferred hand, N=509	258.6 (33.8)

Arithmetic mean (standard deviation) is given for the continuous variables used on their original scale and geometric mean (5th to 95th percentile) for the logarithmically transformed continuous variables. Count (percent) is given for the categorical variables.

[a] Participants for whom information about the covariates, brominated flame retardants blood levels, and at least 1 neurobehavioral measure was available.

[b] At least 1 family member smoking inside the house.

[c] Based on two questions about the amount of warm and cold fish eaten per week. Low corresponds to less than 20 g, middle to 20-25 g, and high to more than 25 g per day.

Blood samples analysis

PBDE congeners 28, 47, 99, 100, 153, 154, 183, and 209, HBCD, and tetrabromobisphenol A (TBBPA) were measured in the serum according to the

method described by Covaci and Voorspoels [53]. Briefly, solid phase extraction was performed to prepare the samples. The eluate was purified on acid silica. The extract was further analyzed by gas chromatography mass spectrometry in electron capture negative ion mode using a 25 m × 0.22 mm × 0.25 μm HT-8 column.

Characteristics of the distributions of the BFRs are shown in Table 2. PBDE congeners 28, 154, and 183 for which less than 5% of the observations had a concentration higher than the limit of quantification (LOQ), were not used in the analysis. For PBDE 47, 99, 100, 153, and 209 binary exposure indicators were used with the LOQ values as thresholds. The logarithm of the sum of PBDEs 47, 99, 100, and 153 was used as a measure of the total long-term PBDE exposure. In the calculation of the sum, the values below the LOQ were replaced by LOQ/2.

Concentrations of polychlorinated biphenyl (PCB) congeners in the serum were determined using the same method as for the BFRs. The sum of PCBs 138, 153, and 180 transformed logarithmically was used as an indicator of the PCB exposure. Lead was measured in the whole blood as described by Schroijen et al. [54]. Blood lipids were measured graphimetrically. Thyroid hormones FT3, FT4, and TSH were measured by competitive immune assays.

TABLE 2. Concentrations of polybrominated flame retardants in serum (ng/L).

	LOQ	Median	P75	P95	Max
BDE28	2	<LOQ	<LOQ	<LOQ	24
BDE47	3	<LOQ	3	9	104
BDE99	3	<LOQ	<LOQ	3	12
BDE100	2	<LOQ	<LOQ	2	42
BDE153	2	2	3	8	24
BDE154	2	<LOQ	<LOQ	<LOQ	6
BDE183	2	<LOQ	<LOQ	<LOQ	5
BDE209	25	<LOQ	<LOQ	53	325
HBCD	30	<LOQ	<LOQ	59	234
TBBPA	15	<LOQ	<LOQ	22	186
SUM PBDE[a]		7	10	21	125

LOQ – limit of quantification.
[a] The sum of PBDE congeners 47, 99, 100, and 153. In the calculation, values lower than the LOQ were replaced by LOQ/2.

Statistical analysis

We used SAS software version 9.2 (SAS Institute Inc, Cary, NC) for all analysis. Continuous positive-value variables with a right-skewed distribution were logarithmically transformed. Normal quantile plots of the residuals were used to examine the normality assumption. For the individual PBDE congeners, HBCD, and TBBPA the effects of the concentrations above the LOQ compared to the concentrations below the LOQ were estimated. The sum of PBDEs was transformed logarithmically and the effect of its two-fold increase was estimated.

In all the models investigating the effects on the neurobehavioral parameters, we corrected for age of the child, gender, type of education (general secondary education versus other), the highest level of education attained by either of the parents (using three indicator variables), whether or not the parents owned the house, smoking, passive smoking, and blood lipids. The remaining covariates were chosen based on a stepwise regression procedure with p=0.15 for entering and p=0.10 for remaining in the model. The considered covariates included BMI of the child, physical activity in leisure time at least once a week, computer use, alcohol use at least once a month, fish consumption, the logarithm of blood lead level, and the logarithm of serum PCB's 138, 153, and 180. In the models investigating the effects of BFRs on FT3, FT4, and TSH levels, we corrected for age, gender, BMI, and blood lipids in all models. Other variables mentioned above besides computer use were included in the model based on a stepwise regression procedure with p=0.15 for entering and p=0.10 for remaining in the model.

Finally, we investigated whether the effects of BFRs were modified by gender by including the interaction term in the regression model.

RESULTS

Characteristics of the study population

The characteristics of the study group are given in Table 1. The adolescents (52.6% boys) were between 13.6 and 17 years of age and the mean age equaled 14.9 years. A majority of the participants (54%) had at least one parent who graduated from a college or a university. A summary of the results obtained in the neurobehavioral tests is given in Table 1.

Determinants of neurobehavioral function

Estimates of the effects of the covariates on the neurobehavioral parameters are shown in Table 3. Gender, age, type of education, parental education, and physical activity were the most important determinants of the performance in the tests.

TABLE 3. Determinants of the neurological parameters.

	Continuous Performance (N=489)			Digit-Symbol (N=340)	Digit Span		Finger Tapping	
	Reaction time (msec)[a]	Errors of omission[b]	Errors of commission[b]	Total latency (sec)[a]	Forward[a] (N=511)	Backward[a] (N=499)	Preferred hand[a] (N=511)	Non-preferred hand[a] (N=509)
Boys		0.43 (0.12)*	0.29 (0.06)*	4.12 (1.82)*			9.71 (3.59)*	5.53 (3.09)
Age, +1 year	-8.57 (2.79)*	-0.14 (0.08)	-0.15 (0.04)*	-5.21 (1.21)*				5.47 (2.2)*
Education type – general	-15.09 (3.76)*	-0.54 (0.11)*	-0.17 (0.06)*	-9.73 (1.82)*	0.44 (0.09)*	0.53 (0.09)*		8.12 (3.05)*
Parents: no diploma[c]							-14.26 (5.7)*	
9 grades[c]								
12 grades[c]					-0.17 (0.1)			
Computer use[d]	-5.26 (2.37)*							
Physical activity	-10.24 (4.57)*						14.72 (4.23)*	7.47 (3.61)*
Blood lead, ×2				3.58 (1.6)*	-0.14 (0.07)			
Sum PCBs, ×2		-0.14 (0.07)*	-0.06 (0.03)		0.14 (0.05)*	0.09 (0.05)	4.44 (2.07)*	4.74 (1.79)*

*p<0.05.
[a] Estimated effect (standard deviation) from a linear model.
[b] Estimated effect (standard deviation) from a negative binomial model.
[c] Reference category: college or university diploma.
[d] The effect of a one level increase of category.
Stepwise regression estimates of the effects of covariates on the neurological parameters with p=0.15 for entering and p=0.10 for remaining in the model. All covariates included in one of the models are shown in the table. The set of considered covariates included: gender, age, BMI, type of education (general secondary education versus other), the highest level of education of parents (three indicator variables), whether or not the parents owned the house, smoking, passive smoking, alcohol use at least once a month, computer use, fish consumption, physical activity in leisure time at least once a week, the logarithm of blood lead, and the logarithm of serum PCB's 138, 153, and 180.

TABLE 4. Estimated effects of serum levels of brominated flame retardants on performance in the Continues Performance, Digit-Symbol, and Digit Span tests.

	Continuous Performance (N=489)			Digit-Symbol (N=340)	Digit Span	
	Reaction time (msec)[a]	Errors of omission[b]	Errors of commission[b]	Total latency (sec)[a]	Forward[a] (N=511)	Backward[a] (N=499)
PBDE47	3.45 (-4.88 to 11.78)	-10% (-29.9 to 15.6%)	6.2% (-6.2 to 20.1%)	-1.19 (-5.57 to 3.2)	-0.09 (-0.29 to 0.11)	-0.07 (-0.27 to 0.14)
PBDE99	-5.39 (-19.85 to 9.08)	-16.3% (-46.4 to 30.8%)	12.9% (-8.6 to 39.5%)	-1.35 (-8.94 to 6.24)	0.09 (-0.25 to 0.44)	0.3 (-0.04 to 0.64)
PBDE100	7.61 (-5.94 to 21.16)	-5.8% (-37.2 to 41.4%)	-3.2% (-21 to 18.5%)	1.98 (-5.6 to 9.56)	-0.26 (-0.57 to 0.06)	-0.18 (-0.49 to 0.14)
PBDE153	5.09 (-2.76 to 12.95)	-19.3% (-36.4 to 2.3%)	-2% (-12.8 to 10.2%)	-1.34 (-5.46 to 2.77)	-0.09 (-0.28 to 0.1)	-0.08 (-0.27 to 0.11)
PBDE209	-1.2 (-14.49 to 12.1)	-17.7% (-45.1 to 23.4%)	1.4% (-16.9 to 23.7%)	2.08 (-4.07 to 8.23)	0.06 (-0.26 to 0.38)	-0.26 (-0.57 to 0.05)
HBCD	-3.53 (-18.72 to 11.67)	27.8% (-17.5 to 97.9%)	21.8% (-2.5 to 52.2%)	-0.44 (-6.59 to 5.72)	0.13 (-0.22 to 0.49)	-0.04 (-0.39 to 0.31)
TBBPA	-2.25 (-17.28 to 12.77)	-9.3% (-43 to 44.2%)	-17.7% (-34.7 to 3.9%)	-2.48 (-10.36 to 5.41)	0.03 (-0.32 to 0.37)	-0.05 (-0.41 to 0.3)
SUM PBDE	2.12 (-2.9 to 7.13)	-6.6% (-19.9 to 8.9%)	0.7% (-6.6 to 8.6%)	-0.39 (-3.04 to 2.26)	-0.01 (-0.13 to 0.11)	-0.04 (-0.16 to 0.08)

Estimated effects and 95% confidence intervals are given.
[a] A linear model.
[b] A negative binomial model (estimated change in %).
For the individual PBDE congeners, HBCD, and TBBPA the effects of levels above the LOQ were estimated. Sum of serum PBDE's 47, 99, 100, and 153 was logarithmically transformed and the effects of its two-fold increase were estimated.
All models were adjusted for gender, age, type of education (general secondary education versus other), the highest level of education of parents (using three indicator variables), whether or not the parents owned the house, smoking, passive smoking, and blood lipids. Additionally, BMI, physical activity in leisure time at least once a week, computer use, alcohol use at least once a month, fish consumption, the logarithm of blood lead and the logarithm of serum PCB's 138, 153, and 180 were included in the model based on the stepwise regression procedure with p=0.15 for entering and p=0.10 for remaining in the model.

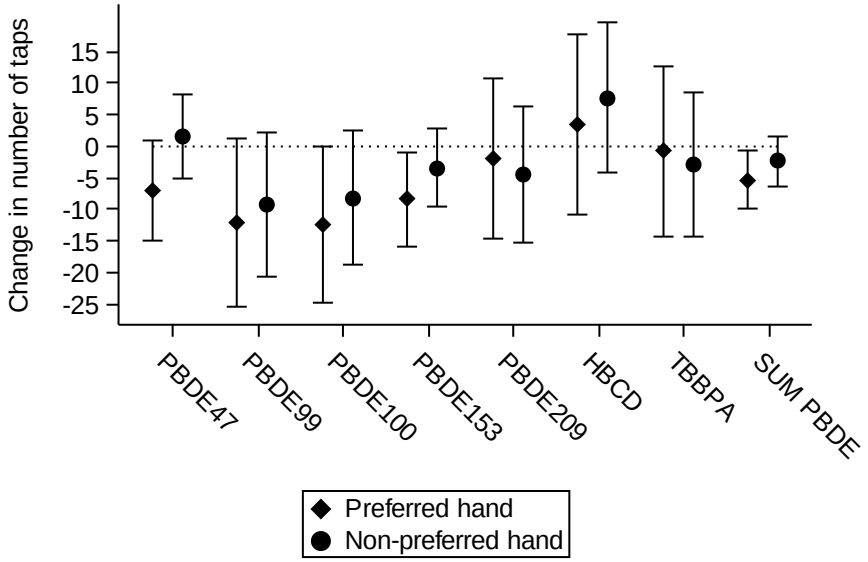

FIGURE 2. Estimated effects of the serum levels of brominated flame retardants on the performance in the Finger Tapping Test.

For the PBDE congeners, HBCD, and TBBPA the effects of levels above the LOQ were estimated. Sum of PBDEs 47, 99, 100, and 153 was logarithmically transformed and the effects of its two-fold increase were estimated. All models were adjusted for: gender, age, type of education (general secondary education versus other), the highest level of education of parents (using three indicator variables), whether or not the parents owned the house, smoking, passive smoking, and blood lipids. Additionally, BMI, physical activity in leisure time at least once a week, computer use, alcohol use at least once a month, fish consumption, the logarithm of blood lead, and the logarithm of serum PCB's 138, 153, and 180 were included in the model based on the stepwise regression procedure with p=0.15 for entering and p=0.10 for remaining in the model.

Associations between BFRs and the neurobehavioral function

We did not find any significant associations between serum levels of BFRs and performance in the Continuous Performance, Digit-Symbol or Digit Span tests (Table 4). However, PBDEs were associated with a deterioration of performance in the Finger Tapping Test in the preferred-hand condition (Figure 2). In the continuous analysis, a two-fold increase of the sum of serum PBDEs was associated with a decrease of the number of taps with the preferred-hand by 5.31 (95% CI: 0.56 to 10.05, p=0.029). The model explained 9.85% of the total variability and 0.87% of the variability could be attributed to the sum of serum PBDEs. Concentrations above the LOQ were associated with an average decrease of 7.04 taps (95% CI: -0.78 to 14.87; p=0.078) for serum PBDE-47, 12.13 (95% CI: -1.30 to 25.57; p=0.078) for serum PBDE-99, 12.43 (95% CI: -0.03 to 24.89; p=0.051) for serum PBDE-100, and 8.43 (95% CI: 1.01 to 15.86; p= 0.026) for serum PBDE-153. The associations between serum PBDEs and the number of taps with the non-preferred hand were usually consistent

(negative association), but did not reach the level of significance. Serum HBCD and TBBPA levels were not significantly associated with performance in the Finger Tapping Test.

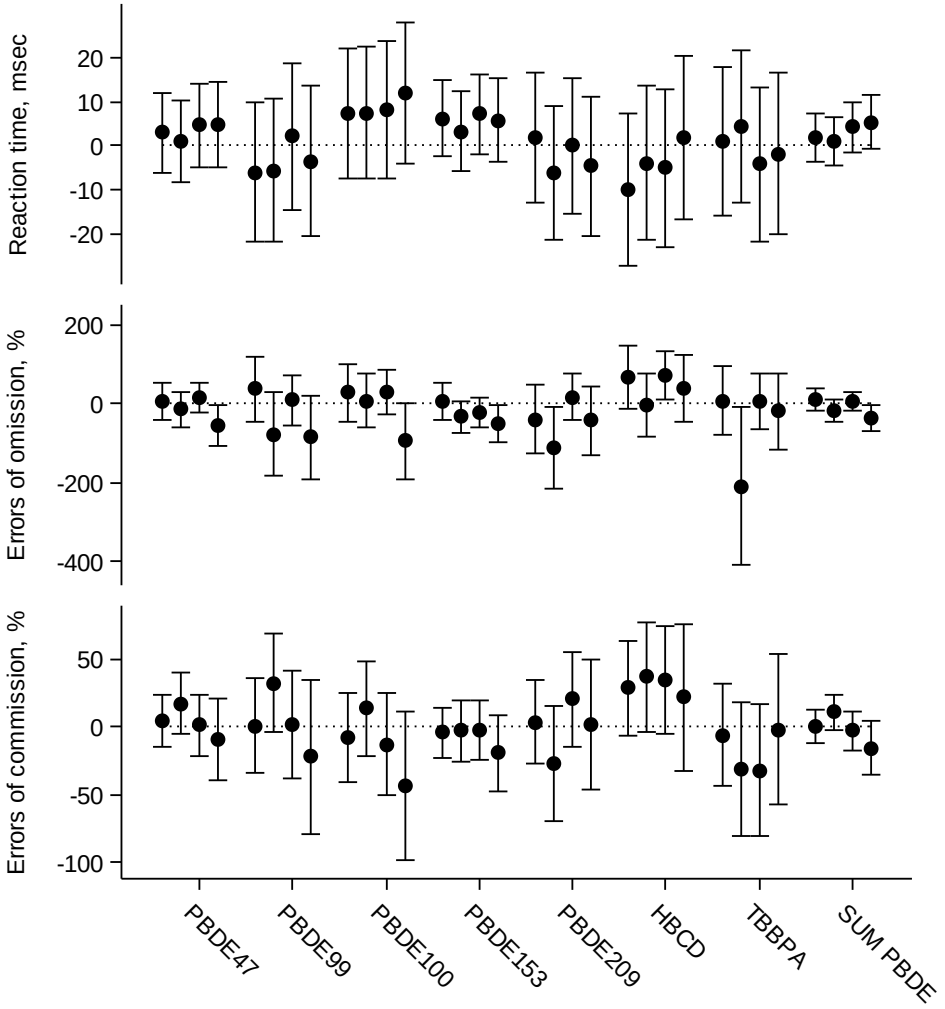

FIGURE 3. Estimated effects of the serum levels of brominated flame retardants on performance in the Continuous Performance Test from analysis with stratification by period.

For each exposure indicator, the effect on outcome in the first, the second, the third, and the fourth block is shown. Each block consisted of 12 trials. The same modeling strategy as in the analysis without stratification was applied.

Also after adjusting for the number of errors of omission and commission, none of the BFRs exposure indicators was significantly related with

the mean reaction time in the Continuous Performance Test. Exposure to BFRs did not show negative associations with performance in the Continuous Performance Test in analysis stratified by period (Figure 3). Gender did not significantly modify the association between the sum of PBDEs and the number of taps with the preferred hand (p=0.25).

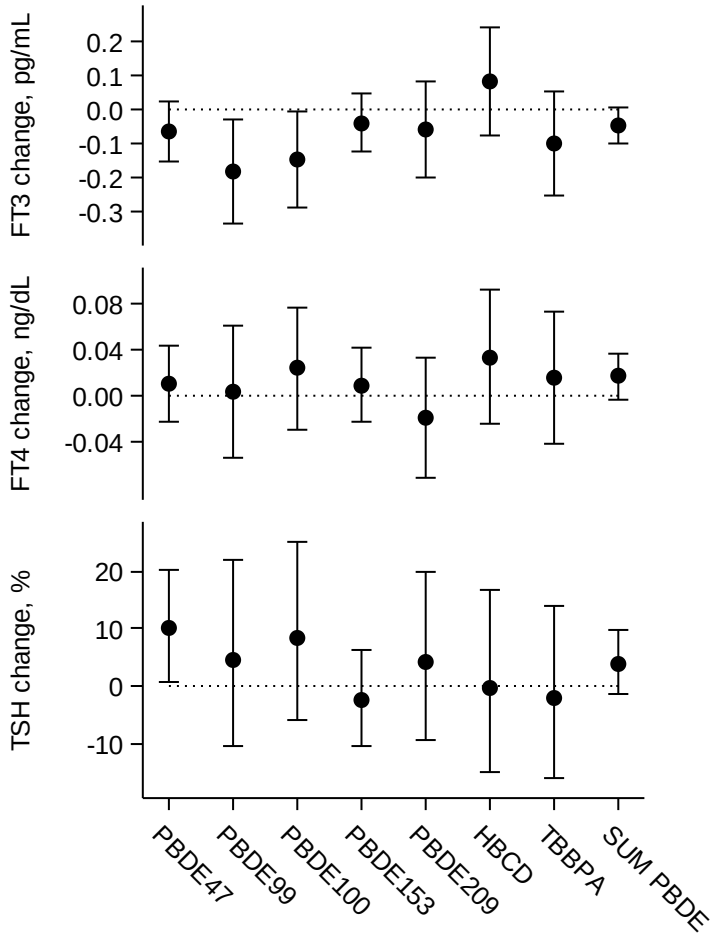

FIGURE 4. Estimated effects of the serum levels of brominated flame retardants on serum FT3, FT4, and TSH concentrations.

For the PBDE congeners, HBCD, and TBBPA the effects of levels above the LOQ were estimated. Sum of PBDEs 47, 99, 100, and 153 was logarithmically transformed and the effects of its two-fold increase were estimated. All models were adjusted for: gender, age, BMI, and blood lipids. Additionally, type of education (general secondary education versus other), the highest level of education of the parents (using three indicator variables), whether or not the parents own the house, smoking, passive smoking, physical activity, alcohol use, fish consumption, BMI, the logarithm of blood lead, and the logarithm of the sum of serum PCB's 138, 153, and 180 were included in the model based on the stepwise regression procedure with p=0.15 for entering and p=0.10 for remaining in the model.

Associations with the FT3, FT4, and TSH levels

The estimated associations between BFRs and FT3, FT4, and TSH serum levels after correction for possible confounders are shown in Figure 4. Serum levels above the LOQ were associated with an average decrease of FT3 level by 0.18 pg/mL (95% CI: 0.03 to 0.34, p=0.020) for PBDE 99 and by 0.15 pg/mL (95% CI: 0.004 to 0.29, p=0.045) for PBDE-100. For the other PBDE congeners the associations had the same direction but were not statistically significant. We did not observe significant associations between PBDE congeners and FT4 levels. PBDE-47 level above the LOQ was associated with an average increase of TSH levels by 10.1% (95% CI: 0.8% to 20.2%, p=0.033). The other PBDE congeners were not significantly associated with TSH.

The continuous analyses did not show significant associations between PBDEs and the thyroid hormone levels. For a two-fold increase of the sum of serum PBDEs, FT3 was estimated to decrease by 0.05 pg/mL (95% CI: -0.01 to 010, p=0.10), FT4 to increase by 0.017 ng/dL (95% CI: -0.003 to 0.032, p=0.10), and TSH to increase by 3.9% (95% CI: -1.5% to 9.6%, p=0.16). We did not observe any significant effects of HBCD or TBBPA on the hormone levels. The adjustment for the levels of the thyroid hormones did not substantially change the estimate of the effect of the sum of PBDEs on the number of taps with the preferred-hand.

DISCUSSION

Consistently with the experimental animal studies demonstrating that exposure to PBDEs during gestation and early childhood affects the motor function [11-22,26-28], we observed negative associations between the serum levels of these BFRs and motor speed of the preferred hand in adolescents. The associations between PBDEs and the second indicator of the motor function, the number of taps with the non-preferred hand, were not significant but showed the same trend. The non-preferred hand task is performed after the preferred hand task in the Finger Tapping Test from the NES3 battery. Our results resemble observations from the experimental animal studies in which a decrease of the motor activity related to PBDE exposure was present only in the beginning of the tests [11,14-21,25]. We did not observe negative associations between BFRs and neurobehavioral domains other than the motor function.

Human data on the neurobehavioral effects of BFRs are scarce. In the United States, a prenatal PBDE exposure was inversely associated with the level of mental development at the age of two and intelligence at the age of two and three [37]. In a Dutch study, a prenatal exposure to PBDE-47 and PBDE-99

showed a negative association with sustained attention and prenatal exposure to PBDE-153 was associated with verbal memory at age five and six [38]. In contrast to these two studies, we conducted a cross-sectional study and focused on older children. The serum levels of PBDEs were not associated with neurobehavioral outcomes in a cross-sectional study of older adults in New York [39]. To our knowledge, our study was the first to investigate the neurobehavioral effects of PBDEs in adolescents.

Higher PBDE-99 and PBDE-100 serum levels were significantly associated with a lower level of serum FT3 and the results for the other PBDE congeners showed the same trend. A negative association between PBDE exposure and the triiodothyronine concentrations was also seen in some other epidemiological studies [55,56]. Consistently with the effects on FT3, most of the indicators of exposure to PBDEs were positively associated with TSH levels, although only for PBDE-47 the level of significance was reached.

Contrary to the experimental animal studies [22,24,41-45], we did not observe a negative association between PBDEs and FT4 concentration. This is in agreement with other epidemiological studies in humans in which either non-significant associations or significant positive associations were reported [56-59]. The discrepancy between animal and human data can be possibly explained by high PBDE doses used in the animal studies in which the effects on thyroxine levels were observed. Although we observed a positive association between PBDE-47 and TSH, the FT4 levels were not negatively associated with PBDEs. A possible explanation is that PBDEs may inhibit the deiodinase enzymes, which serve to metabolize thyroxine to triiodothyronine [59], resulting in an increase in circulating thyroxine and a decrease in circulating triiodothyronine levels.

The biological mechanisms of the effects of PBDEs on the thyroid hormones circulating in the blood have not been fully understood yet. PBDEs exposure caused histological and morphological changes in the thyroid gland in rats indicating its decreased activity [43,60]. It also strongly upregulated uridinediphosphate-glucuronosyltransferase, an enzyme transforming molecules including thyroid hormones into excretable metabolites [44,45]. PBDE exposure also resulted in an induction of pentoxy-resorufin-O-deelthylase activity [42,44,45].

Controlling for the thyroid hormone levels did not substantially change the estimated effects of the sum of PBDEs on the motor speed. Besides the effects on the thyroid function, a disturbance of the cholinergic system may be a pathway by which PBDEs affect the motor function. A neonatal PBDE exposure resulted in reduced or hypoactive behavioral responses to cholinergic agonist nicotine [19,61] and a decreased amount of nicotine receptors in adult rodents [17,62]. PBDEs are also capable to disrupt calcium homeostasis in the brain and cause oxidative stress and apoptotic cell death [9,49].

Elimination of PBDE congener from human tissues depends strongly on the level of bromination [63-65]. The half-life time in human tissues has been

estimated to be around 2 weeks for PBDE-209 [63-65], between a year and a few years for PBDE-47, PBDE-99, and PBDE-100 [63,65], and may be even longer for PBDE-153 [63,65]. Therefore, serum concentration of PBDEs 47, 99, 100, and 153 and the sum of these congeners indicate to a large extent a long-term exposure. The estimated HBCD total body half-life time equaled 64 days [63]. Most of TBBPA was excreted from the body of rat within a few days after administration [66]. The serum BFRs levels at adolescence which we used as exposure indicators, were unlikely to be strongly affected by the exposure during gestation and the first years of life, which may be a period of a particular susceptibility to the neurotoxicity of BFRs.

The main limitation of our study was a large number of observations for which PBDE levels took values below the limit of quantification. In order to deal with this problem, we used binary exposure indicators. However, the limits of quantification which we used to create the categories did not represent critical values separating safe and dangerous exposure levels. This dichotomization of continuous exposure indicators and a possible misclassification due to the use of thresholds which did not have biological relevance might have substantially reduced the statistical power. For 222 participants the levels of both BDE-47, BDE-99, BDE-100, and BDE-153 were lower than the limit of quantification. However, the variability in the sum of these congeners observed in the rest of the participants made it an interesting measure of an overall exposure to PBDEs. Replacing the levels below the LOQ with LOQ/2 in the calculation of the sum introduced some measurement error. This error might have led to an underestimation of the effect of an overall PBDE exposure.

Another disadvantage of our study is that the only aspect of the motor function we investigated was the manual motor speed and that we assessed it only with one test. Although finger tapping is regarded as a reliable measure of motor speed [67,68], a more extended evaluation is needed to verify our findings and investigate the effects of PBDEs on other aspects of the motor function than manual motor speed.

Although we corrected for a number of potential confounders, we cannot exclude that the associations we observed resulted from some source of confounding we failed to account for. Furthermore, a cross-sectional nature of our study does not allow to draw causal conclusions. We cannot exclude the possibility that children with poor motor capabilities chose activities involving BFRs exposure, which resulted in higher blood levels of these toxicants. Similarly, we cannot be sure that the BFRs exposure was a causing factor in the association between the toxicants and the thyroid function we observed. Our study had a fairly low response-rate. However, the comparison of socioeconomical status indicators between a subgroup of participants and non-participants did not reveal evidence of a selection bias.

CONCLUSIONS

Our study is one of few studies and so far the largest one investigating the neurobehavioral effects of BFR in humans. Low-level PBDE exposure was associated with changes in the motor function and serum levels of FT3 and TSH. Our observations need to be further elucidated in other age groups preferably using prospectively designed studies.

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Chapter 3

Neurobehavioral function and low-level metal exposure in adolescents*

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ABSTRACT

An excessive metal exposure is harmful to the brain. However, many aspects of metal neurotoxicity remain unclear including the magnitude of the low-level exposure effects and the level of exposure that can be assumed safe. The aim of our study was to investigate the association between a low-level metal exposure and three neurobehavioral domains (sustained attention, short-term memory, and manual motor speed). We measured Cd, Cu, Mn, Pb, and Tl in blood, Cd, Ni, and toxicologically relevant As in urine and methyl Hg in hair in 606 adolescents between 13.6 and 17 years of age. A two-fold increase in blood Cu was associated with a 0.37 standard deviations decrease in sustained attention (95% CI: -0.67 to -0.07, p=0.02) and 0.39 standard deviations decrease in short-term memory (95% CI: -0.70 to -0.07, p=0.02), accounting for gender, age, smoking, passive smoking, household income per capita, occupation of the parents, and education level of the mother. None of the other metals was significantly associated with the neurobehavioral domains that were measured. The observed associations between blood Cu and neurobehavioral performance are in line with recent studies in elderly. However, the relevance of our results for public health remains to be elucidated.

INTRODUCTION

Excessive exposure to metals has a strong neurotoxic potential. Transition metals copper, manganese, and mercury catalyze redox reactions. Heavy metals lead and cadmium and a metalloid arsenic bind to proteins and interfere with metal transport and protein function and are also capable of inducing oxidative toxicity [1]. Exposure to toxic metals may have serious public health consequences because contaminants such as lead, mercury, and arsenic are widespread. Forty years of epidemiological research has demonstrated that a lead exposure is associated with impaired cognitive function in children [2-4]. There is a growing amount of evidence that lead exposure resulting in blood lead levels lower than 10 µg/dl may affect cognitive performance [5-7]. This raises the issue of changing the current safety threshold [7]. However, it is currently unclear what levels of lead exposure can be assumed safe [8,9].

A high methylmercury exposure has disastrous consequences for the brain, as evidenced by cases of methylmercury poisoning [10-13]. Cohorts from the Faroe Islands and the Seychelles provide information about the neurobehavioral effects of methylmercury exposure resulting from extensive consumption of fish and other seafood from the ocean. In seven years old children from the Faroe Islands, a prenatal methylmercury exposure was associated with a decline in sustained attention (measured using the Continuous Performance Test), short-term memory (the Digit Span Test), manual motor speed (the Finger Tapping Test), verbal learning (the California Verbal Learning Test), and language skills (the Boston Naming Test) [14]. At the age of 14, a prenatal methylmercury exposure was still associated with neurobehavioral performance in this population [15]. However, methylmercury exposure was not associated with neurobehavioral outcomes in children from the Seychelles exposed to methylmercury to a similar extent as those from the Faroe Islands [16]. Results of studies with lower exposure levels were inconsistent [17-20]. Because fish is an important source of essential fatty acids, detailed data on the neurological effects associated with different methylmercury exposure levels is needed in order to decide when a restriction of fish consumption due to methylmercury contamination is advisable.

Arsenic, a potent toxicant, can also cause injury to the central nervous system [21]. In children highly exposed through contaminated groundwater, the urine levels of this metal were negatively associated with cognitive performance [22-24]. The neurobehavioral effects of a low-level exposure to arsenic remain largely unexplored.

Some [25-27] but not all [28,29] studies on cadmium exposure and cognition revealed negative associations. Human cases of poisoning and animal studies have showed that nickel [30,31] and thallium [32,33] are neurotoxic at

high exposure levels. However, little is known about the neurobehavioral effects of these heavy metals at a low level of exposure.

Essential nutrients manganese [34,35] and copper [36,37] are neurotoxic at high doses. As both deficiency and excess of these elements can be harmful, the optimal levels need to be carefully determined. This is difficult at this moment due to the scarcity of data.

Here, we investigated the association of metal exposure with sustained attention, short-term memory, and manual motor speed in 606 adolescents.

MATERIALS AND METHODS

Study population and data collection

The study population is described in detail elsewhere [38]. Briefly, the study was a part of a biomonitoring program for environmental health surveillance in Flanders, Belgium. Between 2008 and 2011, we recruited third year secondary school students in two industrial areas, Genk (n=197) and Menen (n=199), and from the general population of Flemish adolescents (n=210). In the industrial areas, all pupils living within the selected study area who spoke Dutch were eligible. A multistage sampling was performed to select participants from the general Flemish population.

Two weeks before the study, participants received two questionnaires to fill in, one for themselves and one for their parents. The questionnaire for adolescents included questions about their smoking behavior. Questions about education level, income, and occupation were included in the questionnaire for parents. Each participant received a plastic bottle and was asked to collect a first morning urine sample at the day of the neurobehavioral examination. During the study session, the neurological tests were administered and blood and hair samples were collected. The samples were transported to the laboratory in a cool box and subsequently stored at -20^0C until analysis. A team of nurses and other health-care personnel with years of experience at biomonitoring programs involving neurobehavioral testing and collecting of biological samples performed the examinations. An experienced psychologist Griet Vermier trained that team on how to administer the NES tests. Both parents and teenagers provided informed consent for participation. The study was approved by the Ethical Committee of the University of Antwerp.

Exposure indicators

100 µL distilled nitric acid was added to 500 µL urine to eliminate the organic matrix. Afterwards, the samples were diluted with Milli Q water. Urine Cd

and Ni levels were determined using Inductively Coupled Plasma-Mass Spectrometry [39]. The method was validated using certified control materials (Seronorm Trace Elements Urine and Lyphochek Controls for Trace Metals Testing). As external quality control, we participated in ring tests of the German External Quality Assessment Scheme. Toxicologically relevant arsenic concentrations in the urine were defined as the sum of inorganic arsenic, monomethylarsonic acid, and dimethylarsinic acid and measured using Flow-Injection Hydride Generation Atomic Absorption Spectrometry [40]. The method was validated using certified reference materials (Seronorm Trace Elements Urine and ClinChek® Urine Controls). As external quality control, we participated in ring tests of the German External Quality Assessment Scheme. The metals measured in the urine were expressed per gram creatinine.

Cd, Cu, Pb, Mn, and Tl concentrations in the whole blood were measured using Inductively Coupled Plasma-Mass Spectrometry [41]. The method was validated using certified reference samples (Sero A.S.). As external quality control, we participated in ring tests of the German External Quality Assessment Scheme. Headspace-Gas Chromatography-Atomic Fluorescence Spectrometry was applied to measure the hair methyl Hg levels [42]. We validated this method using blanks, samples spiked at difference levels, and certified reference materials (IAEA-086).

The level of Ni in the urine was lower than the limit of detection (LOD) for five participants. All participants had detectable levels of the remaining metals.

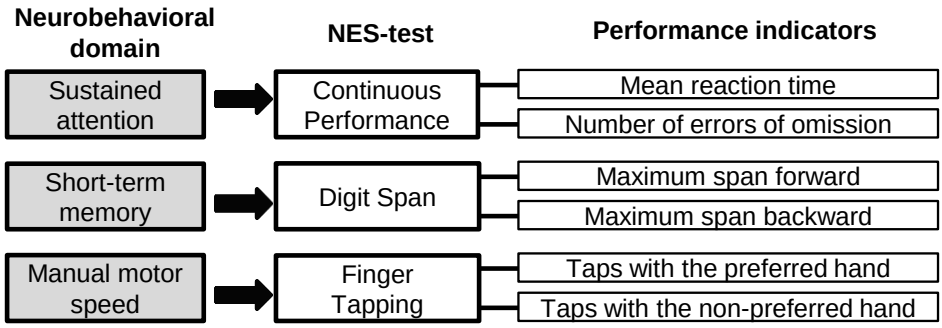

FIGURE 1. Overview of the neurobehavioral outcomes. NES: Neurobehavioral Evaluation System.

Neurobehavioral outcomes

The Neurobehavioral Evaluation System (NES) is a computer-administered battery of tests that was developed to study the neurological effects of exposure to environmental agents [43] and has been commonly utilized for this purpose [14,44-46]. In this study, we administered the

Continuous Performance, Digit Span, and Finger Tapping tests from the NES3 version of the battery [47] (Figure 1). We chose these tests because they measure important neurobehavioral domains. Moreover, studies observing a negative association between neurotoxicants and results in these tests [14,15,48,49] suggested that the tests were sensitive to the effects of environmental pollution.

In the Continuous Performance Test, a series of letters was displayed on the screen. The task was to immediately respond to the letter S but not to other letters. A new letter appeared each 1000msec and remained on the screen for approximately 200msec. In total, the letter S appeared 60 times and the last 48 appearances were used to calculate the performance indicators. We used the mean reaction time for correct responses and the number of errors of omission (i.e. the number of non-responses) as indicators of sustained attention. The Continuous Performance Test is a validated and widely used measure of sustained attention [50]. In the clinical context, the validity of the test is supported by a weaker performance of individuals with a traumatic brain injury [50] and ADHD [51-53].

The Digit Span Test consisted of two parts. In the first part, the task was to reproduce sequences of digits after an auditory presentation. In the second part, the sequences were reproduced in the reverse order. The maximum span forward and backward were used as indicators of short-term memory. The Digit Span Test is a validated and commonly used measure of short-term memory [54], which is an important predictor of cognitive functioning [55-57].

In the Finger Tapping Test, a subject pressed the spacebar as many times as possible during a trial of 10 seconds. The first part of the test consisted of four trials with the preferred-hand. The second part consisted of four trials with the non-preferred hand. We used the total number of taps with the preferred and the total number of taps with the non-preferred hand as indicators of manual motor speed. The Finger Tapping Test is a common way to assess fine motor speed [58]. The clinical validity of this test has been demonstrated by studies comparing patients with movement disorders to controls [59,60].

Statistical analysis

All exposure indicators were right-skewed. In order to reduce the skewness, we transformed them logarithmically. We standardized (subtracted the mean and divided by the standard deviation) all continuous neurobehavioral parameters to express them on the same scale. For each combination of exposure indicator and neurobehavioral domain, we used a separate multivariate model. For sustained attention, we applied bivariate latent variable models for clustered continuous (the mean reaction time in the Continuous Performance Test) and discrete (the number of errors of omission in the Continuous Performance Test) outcomes [61,62]. The parameters were estimated by

assuming that the number of errors of omission had a corresponding unobserved latent continuous variable and that this latent variable and the mean reaction time shared a bivariate normal distribution. For short-term memory, we used bivariate ordinal probit models with two discrete outcomes (digit span forward and backward) [63]. In this case, each indicator of short-term memory was assumed to have an underlying latent variable that shared a joint normal distribution. For manual motor speed, we applied bivariate regression models with two continuous outcomes (number of taps with the preferred and non-preferred hand) [64]. For example, in the case of sustained attention, the following model was used:

$$Y_{ij} = \alpha_i + \beta_{exp} exposure_j + \beta_1 X_{1j} + \cdots + \beta_p X_{pj} + \varepsilon_{ij},$$

where Y_{1j} is the mean reaction time in the Continuous Performance Test for person j, Y_{2j} is a continuous latent variable underlying the number of errors of omission for person j, α_i are intercepts, $exposure_j$ is exposure indicator, and X_{1j},..., X_{pj} are covariates for person j.

For Cu and Mn, quadratic models were applied in order to model the possibly non-linear associations between these elements and neurobehavioral function (i.e., the term $\beta_{qu} exposure_j^2$ was added to the model). All multivariate models allowed for a correlation between the neurobehavioral indicators. We used Bayesian multiple imputation to handle missing values [65]. We created 50 versions of the complete data, in which all missing values were replaced by plausible values. The imputation model included all variables used in the analysis. The analysis was performed for each version of the complete data and the obtained estimates were combined using the Rubin's rules [65,66]. As potential confounders, we considered gender, age, smoking, passive smoking, household income per capita, the highest occupational category of either parent, and the education level of the mother. We modeled smoking and passive smoking with indicator variables. Adolescents who smoked at least once a month were categorized as smokers. Adolescents having at least one family member smoking inside the house were categorized as passive smokers.

In sensitivity analysis, we investigated whether gender was a modifier of the associations between metals and neurobehavioral performance. Additionally, we examined a possible confounding of ferritin, time of the examination, and parents' country of birth by adding these covariates to the model. A possible confounding of time of the examination was investigated by adding restricted cubic spline functions based on three knots located at the 5th, 50th, and 95th percentile. Additionally, we investigated whether binary indicators of exposure to Pb, methyl Hg, toxicologically relevant As, and Cd (>90th percentile versus not) were associated with neurobehavioral function. We used SAS software version 9.3 (SAS Institute Inc, Cary, NC) for all analyses.

TABLE 1. Descriptive statistics.

N=606	
Demographic characteristics, N=606[a]	
Boys	324 (53.5%)
Age	14.9 (0.7)
Both parents born outside Belgium, N=595[a]	51 (8.6%)
Level of education of the mother, N=550[a]	
no diploma	38 (6.9%)
9 grades	72 (13.1%)
12 grades	190 (34.5%)
College or university diploma	250 (45.5%)
Highest category of occupation of either parents, N=559[a]	
Unemployed or not qualified worker	44 (7.9%)
Qualified worker, white-collar assistant or teaching staff	267 (47.8%)
Self-employed, specialist or member of management	248 (44.4%)
Household income per capita [euro], N=535[a]	750 (296)
Smoking, N=602[a]	46 (7.6%)
Passive smoking, N=598[a]	105 (17.6%)
Examination time, N=606[a]	
Before 10am	224 (37%)
Between 10am and the noon	354 (58.4%)
Between the noon and 2pm	28 (4.6%)
Serum ferritin [µg/L], N=602[a]	35.3 (22.9)
Neurobehavioral parameters	
Continuous Performance, reaction time [msec], N=535[a]	410 (42)
Continuous Performance, errors of omission [number], N=535[a]	2.3 (2.67)
Digit Span Forward [number of digits], N=597[a]	5.57 (1.04)
Digit Span Backward [number of digits], N=579[a]	4.48 (1.03)
Finger Tapping, preferred hand [number of taps], N=597[a]	294 (42)
Finger Tapping, non-preferred hand [number of taps], N=595[a]	259 (35)

Arithmetic mean (standard deviation) is given for the continuous variables. Count (percent) is given for the categorical variables.

[a] Number of participants for whom information was available.

RESULTS

Characteristics of the study population

Descriptive statistics of the demographic and lifestyle variables and the neurobehavioral parameters are presented in Table 1. The study included 324 boys and 282 girls between 13.6 and 17 years of age. The mother of almost half of the children graduated from a college or a university. A small proportion of the children (7.9%) had both parents who were unqualified workers or unemployed. The mean concentration was 13.8 µg/L for blood Pb, 9.9 µg/L for blood Mn, and 821 µg/L for blood Cu (Table 2). The mean urine toxicologically relevant As level was 3.7 µg/g creatinine. The mean hair methyl Hg concentration equaled 0.11 µg/g. The proportion of missing observations was smaller than 14% for all variables (Table 1 and Table 2).

TABLE 2. Descriptive statistics of the exposure indicators.

	N	Mean[a]	5th Pctl	25th Pctl	Median	75th Pctl	95th Pctl	Refer. values
Blood Cd, µg/L	606	0.21	0.10	0.15	0.20	0.27	0.58	0.16[b]
Blood Cu, µg/L	606	821	659	747	815	887	1035	637-1401[c]
Blood Mn, µg/L	606	9.9	6.3	8.1	9.9	11.8	15.4	9.9[b]
Blood Pb, µg/L	606	13.8	7.3	10.5	13.6	17.6	28.1	5.5[b]
Blood Tl, µg/L	606	0.030	0.020	0.026	0.030	0.035	0.045	0.020[d]
Urine tox. rel. As, µg/g crt	605	3.7	1.2	2.7	3.7	5.6	13.7	4.7[b,e]
Urine Cd, µg/g crt	533	0.21	0.10	0.16	0.21	0.28	0.71	0.07[f]
Urine Ni, µg/g crt	533	1.62	0.61	1.11	1.60	2.36	7.01	0.88[d]
Hair Methyl Hg, µg/g	580	0.11	0.02	0.07	0.12	0.21	0.47	0.13[g]

Pctl: percentile, crt: creatinine.
[a] Geometric mean.
[b] Median concentration in adolescents between 12 and 19 years of age examined in 2011 and 2012 in the USA [67].
[c] Normal blood copper levels [68].
[d] Median concentration in healthy British citizens without occupational exposure between 16 and 70 years of age (data from 1998) [69].
[e] Total arsenic.
[f] Geometric mean for adolescents between 12 and 19 years of age examined in 2007 and 2008 in the USA [70].
[g] Geometric mean in American girls between 16 and 19 years of age (data from 1999-2000) [71].

Associations between metals and neurobehavioral function

Cd, Pb, and Tl in the blood, toxicologically relevant As, Cd, and Tl in the urine, and methyl Hg in the hair were not significantly associated either with sustained attention ($p>0.10$), short-term memory ($p>0.13$), or manual motor speed ($p>0.10$, Figure 2).

As both deficiency and excess of Cu and Mn may have a negative impact on neurobehavioral function, we considered quadratic models for these essential nutrients. However, because we found no evidence that the associations were quadratic ($p>0.11$), we report estimates from linear models. A two-fold increase in blood Cu was associated with a 0.37 standard deviations decrease in sustained attention (95% CI: -0.67 to -0.07, $p=0.02$) and 0.39 standard deviations decrease in short-term memory (95% CI: -0.70 to -0.07, $p=0.02$, Figure 2). Parameter estimates from these models for all covariates are available in Appendix A. The association between blood Cu and manual motor speed was not statistically significant ($p=0.16$). There was no evidence of an association between blood Mn and the neurobehavioral domains ($p>0.10$, Figure 2).

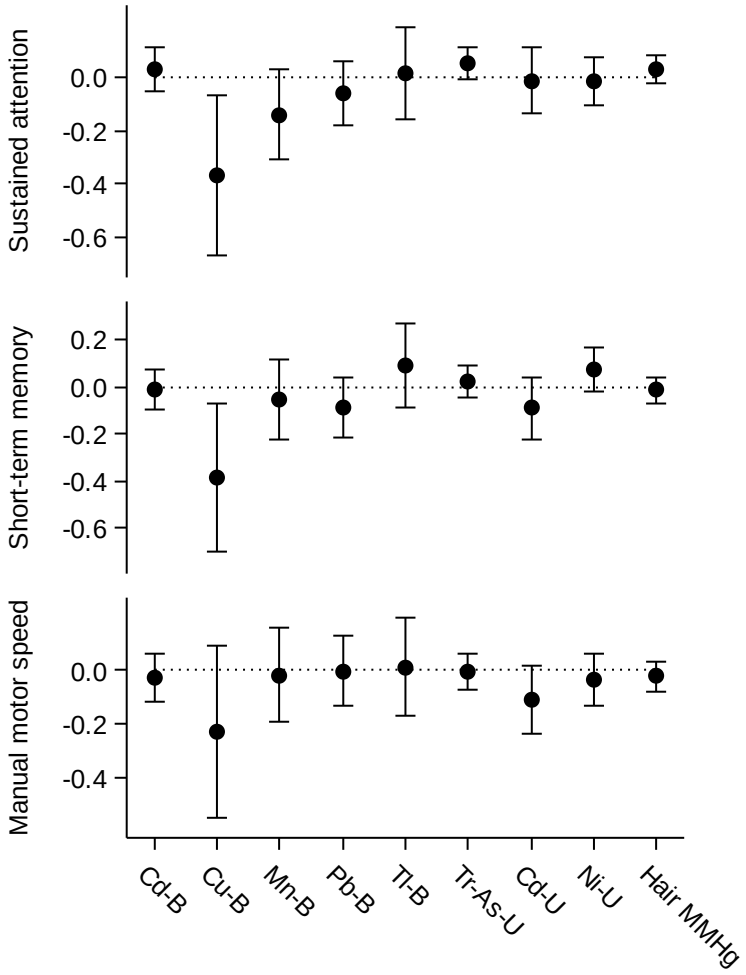

FIGURE 2. Associations between metal exposure and the neurobehavioral outcomes.

Estimates of the number of standard deviations change in the neurobehavioral domains associated with a two-fold increase in the level of metal and confidence intervals are presented. All models accounted for gender, age, smoking, passive smoking, household income per capita, the highest occupational category of either parent, and the education level of the mother.

Sensitivity analysis

The effect modification of gender was not statistically significant for any of the metals for any neurobehavioral domain ($p>0.14$ for sustained attention, $p>0.16$ for short-term memory, and $p>0.10$ for manual motor speed). However, a subgroup analysis revealed an association between blood Cu and neurobehavioral outcomes in girls and no evidence of an association in boys (Figure 3). In girls, a two-fold increase in blood Cu was associated with a 0.54

standard deviations decrease in sustained attention (95% CI: -0.92 to -0.15, p=0.01) and 0.52 standard deviations decrease in short-term memory (95% CI: -0.92 to -0.12, p=0.01). Because the phase of menstrual cycle was a possible confounder of the association between blood Cu and cognitive outcomes in girls, we performed additional analysis including an indicator variable for menstruation at the examination day (yes versus no). Accounting for menstruation did not change the estimates more than by 0.01 neither for attention nor for short-term memory.

The serum level of ferritin, time of the examination, and parents' country of birth were not important confounders of the associations between metals and the neurobehavioral domains. The association of blood Cu with sustained attention and short-term memory remained statistically significant and the estimates did not change more than by 0.03 standard deviations after an inclusion of one of these covariates in the models. For the remaining exposure indicators, an inclusion of the serum level of ferritin, time of the examination, or parents' country of birth as predictors of sustained attention, short-term memory, or manual motor speed did not change the estimates by more than 0.02 standard deviations. We did not find evidence of an association between the categorical indicators of exposure to blood Pb, methyl Hg, toxicologically relevant As, or Cd (>90th percentile versus not) and the neurobehavioral domains.

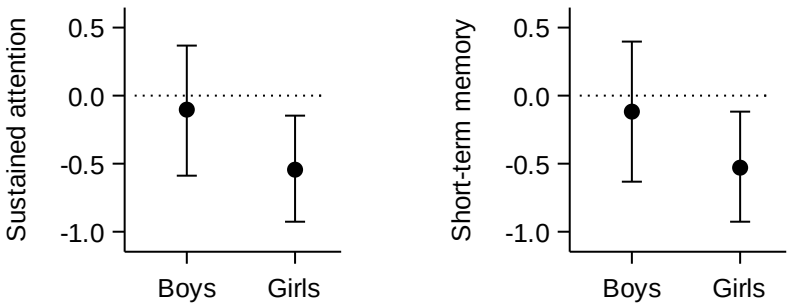

FIGURE 3. Associations between blood copper and sustained attention and short-term memory in boys and girls.

Estimates of the number of standard deviations change in the neurobehavioral domains associated with a two-fold increase in the level of copper and confidence intervals are presented. All models accounted for age, smoking, passive smoking, household income per capita, the highest occupational category of either parent, and the education level of the mother.

DISCUSSION

Blood copper was associated with sustained attention and short-term memory in a population of adolescents with normal copper levels. Stratified analysis for gender revealed significant associations between copper and neurobehavioral performance only for girls. For the other studied metals and arsenic, we did not find evidence of an association with sustained attention, short-term memory, or manual motor speed. A possible explanation of these results is that the neurotoxic effects exerted at exposure levels characterizing our population were too small to allow observing changes in neurobehavioral performance in adolescents. In general, the concentrations of metals and As that we observed were similar as in other recent Western studies (Table 2). A number of studies observed a negative association between lead and intelligence at blood levels below 10 μg/dl [5,6,72]. However, the typical current blood lead levels in our population are much lower than 10 μg/dl. In our study group, 95% of the adolescents had blood lead levels lower than 2.81 μg/dl and 75% lower than 1.76 μg/dl. The hair level of methylmercury was approximately 10-fold lower in our population than in children from the Faroe Islands, in whom methylmercury was associated with several neurobehavioral domains including sustained attention, short-term memory, and manual motor speed [14]. Also the urinary level of arsenic was much lower in our population than in children exposed to highly contaminated water, in whom arsenic was associated with neurobehavioral performance [22-24].

Although epidemiological data investigating the cognitive effects of a low-level copper exposure are scarce, our study is not the first one suggesting that the concentrations regarded as normal may actually affect cognition. Two cross-sectional studies showed a negative association between cognitive performance and plasma copper in elderly [25,73]. A prospective cohort study reported a strong association between copper intake and cognitive decline over a period of six years among 604 elderly with a diet high in fat [74]. Alzheimer's disease patients have been reported to have a higher serum copper level than elderly without neurological disorders [75].

Copper is an essential trace element involved in redox reactions such as cytochrome oxidase, ascorbate oxidase, and superoxide dismutase. A disruption of ion homeostasis leads to oxidative stress. Copper has a strong potential to catalyze a creation of reactive oxygen species such as the hydroxyl radical through the Fenton reaction [21]. Copper crosses the blood-brain barrier and is distributed throughout most regions of the brain. In a clinical context, dramatic neurological consequences of its oxidative potential have been evidenced in patients with Wilson disease, an autosomal recessive disorder resulting in an accumulation of copper in tissues including the brain [76].

Importantly, in healthy people the rate of copper excretion changes according to the copper intake [76]. Therefore, copper exposure measured by the blood levels only partly reflects the amount of copper intake, and is largely determined by individual differences in copper metabolism. Both slow metabolism and high intake may be responsible for the neurological effects of copper. The role of excessive intake was supported by recent experimental animal studies. Dietary copper induced an accumulation of beta-amyloid and caused cognitive impairments in mice and rabbits with a diet rich in cholesterol [77,78]. Copper treatment exacerbated beta-amyloid and tau pathologies in a mouse and rat model of Alzheimer's disease [79,80].

The levels of blood copper may fluctuate during the menstrual cycle reaching high values during menstruation [81]. Several studies have reported a poorer cognitive performance of eumenorrheic women in the menstrual phase [82,83]. Therefore, the inverse association between blood copper and cognitive outcomes observed in girls could possibly be explained by hormonal changes related to the menstrual cycle, and not by copper neurotoxicity per se. However, accounting for menstrual phase did not modify the estimates suggesting that it was not an important confounder in our study. Another plausible explanation of the gender-related differences is that estrogen promotes retention of copper [84,85]. As a result, girls may be particularly susceptible to the toxic effects of copper.

Our study has several limitations. It has been suggested that particularly the level of serum free copper - copper not bound to ceruloplasmin – is linked to the cognitive function [86]. Unfortunately, we only measured total copper. Another limitation is that only one measurement was available. This might be insufficient to adequately reflect the exposure over time to some of the metals including lead. A major strength of our study is the simultaneous use of multiple indicators to measure the neurobehavioral outcomes, which is likely to substantially increase the statistical power [87-89].

Since the amount of human data on the association between blood copper and cognition is scarce, our results in a group of adolescents do not have any practical implications at the moment. However, they add to the recent evidence based on elderly and other adults suggesting that copper may be relevant for neurobehavioral function at copper levels regarded as normal. Future research should further investigate whether copper exposure affects neurobehavioral outcomes, and if so, clarify in what circumstances. Our study did not provide evidence of neurotoxicity from a low-level exposure to lead, methylmercury, arsenic, manganese, cadmium, nickel, or thallium.

APPENDIX A. PARAMETER ESTIMATES

	Sustained attention		Short-term memory	
	Effect size	95% CI	Effect size	95% CI
Boys	-0.26	-0.40 to -0.13	-0.08	-0.23 to 0.06
Age, +1 year	0.21	0.10 to 0.31	-0.01	-0.12 to 0.10
Education of the mother[a]				
no diploma	-0.49	-0.83 to -0.15	-0.55	-0.90 to -0.19
9 grades	-0.04	-0.27 to 0.20	-0.28	-0.53 to -0.03
12 grades	-0.23	-0.39 to -0.06	-0.30	-0.48 to -0.12
Occupation of the parents[b]				
Unemployed or not qualified worker	-0.08	-0.38 to 0.22	0.12	-0.20 to 0.43
Qualified worker, white-collar assistant, teaching staff	-0.04	-0.19 to 0.11	-0.12	-0.27 to 0.04
Income, +IQR	0.01	-0.10 to 0.13	0.01	-0.11 to 0.14
Smokers	-0.11	-0.37 to 0.15	0.04	-0.23 to 0.32
Passive smokers	0.02	-0.17 to 0.20	0.03	-0.17 to 0.22
Blood Cu, a 2-fold increase	-0.37	-0.67 to -0.07	-0.39	-0.70 to -0.07

The table shows the estimated number of standard deviations change in the neurobehavioral domains. Negative coefficients indicate a negative association with neurobehavioral performance.

[a] Reference category: college or university diploma.

[b] Highest category of occupation of either parents. Reference category: self-employed, specialist or member of management.

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Chapter 4

Neurobehavioral performance in adolescents is inversely associated with traffic exposure*

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ABSTRACT

On the basis of animal research and epidemiological studies in children and elderly there is growing concern that traffic exposure may affect the brain. The aim of our study was to investigate the association between traffic exposure and neurobehavioral performance in adolescents. We examined 606 adolescents. To model the exposure, we constructed a traffic exposure factor based on a biomarker of benzene (urinary *trans*,*trans*-muconic acid) and the amount of contact with traffic preceding the neurobehavioral examination (using distance-weighted traffic density and time spent in traffic). We used a Bayesian structural equation model to investigate the association between traffic exposure and three neurobehavioral domains: sustained attention, short-term memory, and manual motor speed. A one standard deviation increase in traffic exposure was associated with a 0.26 standard deviations decrease in sustained attention (95% Bayesian credible interval: -0.02 to -0.51), adjusting for gender, age, smoking, passive smoking, level of education of the mother, socioeconomic status, time of the day, and day of the week. The associations between traffic exposure and the other neurobehavioral domains studied had the same direction but did not reach the level of statistical significance. The results remained consistent in the sensitivity analysis excluding smokers and passive smokers. The inverse association between sustained attention and traffic exposure was independent of the blood lead level. Our study in adolescents supports the recent findings in children and elderly suggesting that traffic exposure adversely affects the neurobehavioral function.

INTRODUCTION

There is a growing public health concern that traffic-related air pollution may be harmful to the brain [1]. Inhaled ultrafine particles translocate to the brain [2,3] and are able to induce oxidative stress in the neuronal cells [4,5]. Exposure to traffic-related air pollution increases pro-inflammatory mediators in the systemic circulation [6], which affects the brain function [7,8]. In line with these results, multiple experimental studies in rodents have shown that airborne particulate matter increases biomarkers of neuroinflammation [9,10], which may lead to a deterioration in cognitive performance and play a crucial role in the development of neurological disorders [11,12]. Neurotoxic changes have also been observed in humans exposed to air pollution [13,14]. Studies comparing children living in a city with much air pollution to those living in clean areas revealed that exposure to air pollution was associated with a deposition of particulate matter in bulb neurons, neuroinflammation (as indicated by an up-regulation of cyclooxygenase-2, interleukin-1β, and monocyte chemoattractant protein), low concentrations of cytokines involved in neuroprotection, and accumulation of amyloid β42 [13,14].

A number of recent studies have observed a negative association between air pollution exposure and neurobehavioral outcomes. Average lifetime concentrations of residential black carbon were negatively associated with intelligence in children between 8 and 11 years of age [15] and with sustained attention in children between 7 and 14 years of age [16]. In two independent cohorts, children who had a higher prenatal exposure to ambient polycyclic aromatic hydrocarbons as evaluated by personal air monitoring of the mothers showed a lower IQ at the age of five [17,18]. In elderly, a higher particulate matter exposure was associated with a decline in cognitive performance [19-22]. To date, the neurobehavioral effects of traffic exposure in age categories other than young children and elderly have received little attention.

A study of 200 Flemish adolescents showed that those living in suburbs crossed by busy highways (>80,000 vehicles per day) had higher urinary levels of *trans,trans*-muconic acid (*t,t*-MA-U) than those living in a control area with little traffic [23], thus highlighting the usefulness of this metabolite of benzene as a proxy-biomarker for traffic exposure. Several other studies have shown that exposure to vehicle exhaust is associated with an increase in the *t,t*-MA-U levels [24-26].

Here, we investigated in another group of Flemish adolescents whether traffic exposure may affect sustained attention, short-term memory, and manual motor speed. A major challenge in studies of health effects of traffic-related air pollution involves accurate assessment of the exposure. In order to address this challenge, we applied structural equation modeling, which allowed us to utilize

different sources of information about the exposure including traffic density, time spent in traffic, and *t,t*-MA-U.

MATERIALS AND METHODS

Study population and data collection

A detailed description of the study population can be found elsewhere [27]. Briefly, as part of a biomonitoring program for environmental health surveillance in Flanders, Belgium, we recruited between 2008 and 2011 grade nine high school students in two specific areas, Genk (n=197) and Menen (n=199), and from the general population of Flemish adolescents (n=210). Genk and Menen were selected due to a high level of industrial activities in these cities and their surroundings. Only adolescents who spoke Dutch were eligible. In Flanders, a region of Belgium with an area of only 13,522 km^2 and approximately 6 million inhabitants, the number of vehicles exceeded 3.7 million in 2009 [28]. In the same year, these vehicles travelled 56.4 billion km [28]. With a dense network of roads including over 800 km highways, 6200 km other major roads, and over 64,000 km secondary roads [28], every resident is exposed to traffic at least to some extent.

Two weeks before the study, participants and their parents received questionnaires to fill out. The questionnaire for adolescents included questions about their smoking behavior. The questionnaire for parents included questions about their education level, income, occupation, and the amount of time spent in traffic by their children. We asked how many minutes the child spends per day travelling by car, bus, or tram during the week and during the weekend. The high correlation between the amount of time spent in traffic by adolescents during a weekday and the logarithm of the distance between the school and home locations (r=0.52) confirmed the validity of the questionnaire. Each participant received a plastic bottle and was asked to collect a first morning urine sample at the day of the neurobehavioral examination. However, 1.3% of the participants collected a urine sample in the evening preceding the neurobehavioral examination and for 19.3% of the adolescents a urine sample was collected at school during the examination. For most participants (85%), the urine sample was collected not more than four hours before the administration of the neurobehavioral tests. The samples were stored in a cooler (4^0C) during transportation and kept frozen at -20^0C until analysis. Both parents and teenagers provided informed consent for participation. The study was approved by the Ethical Committee of the University of Antwerp.

Distance-weighted traffic density (DWTD)

We constructed a DWTD measure, which assumes that airborne exhaust pollutants spread in a Gaussian manner [29-31]. In this approach, the impact of traffic is modeled as a function of the traffic density and the distance to the road according to the following equation:

$$w = \frac{1}{0.4*\sqrt{2\pi}} exp\left[-\frac{1}{2} * \frac{\left({}^{D}/_{150}\right)^{2}}{(0.4)^{2}}\right],$$

where *D* is the distance to the road in meters and *w* the corresponding weight factor (Figure 1). The shape of the equation reflects the assumption that 96% of the emissions disperse at 150 meters from the road, which is based on research that investigated the dispersion of vehicle exhaust pollutants [32]. We calculated DWTD for each school and home location by adding up the traffic density in the neighborhood weighted by the distance (Figure 1). The resulting values were log transformed to normalize the distribution. The length of the roads was calculated using the Geographical Information System (GIS, ArcMap version 10.0). Information about the average number of vehicles on highways and other major roads was available from a network of measuring stations run by the Department of Mobility and Public Works. In order to estimate the number of vehicles on not major roads, we used information about their total length and the number of kilometers travelled on these roads [28], which resulted in the average of 543 cars per day.

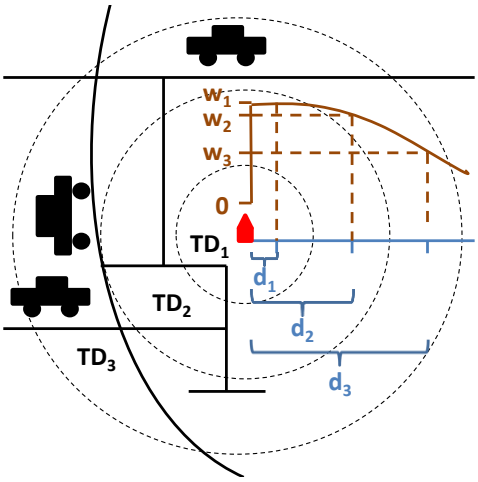

FIGURE 1. Distance-weighted traffic density (DWTD).

For each location, $DWTD=TD_1w_1+TD_2w_2+\ldots+TD_{50}w_{50}$, where TD_i is the traffic density within ring *i* and w_i is the corresponding weight, *i*=1, …, 50. The traffic density is expressed as the total number of kilometers travelled by all vehicles during one day. We calculated traffic density for each road by multiplying traffic counts (the number of cars passing a measuring station during one day) by the length of the road. w_i is a value of a Gaussian function for d_i, where d_i is the distance to a middle point of ring *i*. We used d_1=5 m, d_2=15 m, d_3=25 m, …, d_{50}=495 m, which produced accurate results within a relatively short computation time. For *i*>50, TD_iw_i equaled approximately 0.

trans,trans-muconic acid in urine

We used the level of *t,t*-MA-U as a proxy-biomarker of traffic exposure. We used the method of Angerer et al. for the measurements [33]. Previous research showed that urine could be stored longer than one year at -20 ^{0}C [33]. To ensure reliability of the *t,t*-MA-U measurements, we analyzed the samples within the year after sample collection. *t,t*-MA was isolated from the urine using ion-exchange chromatography. After elution with acetic acid (10%), we separated *t,t*-MA from the remaining components with High Performance Liquid Chromatography and applied a diode array detector. For 603 out of 606 participants, the concentration of *t,t*-MA-U was higher than the limit of detection (5 μg/L). We expressed the level of *t,t*-MA-U per gram creatinine and transformed it logarithmically. *t,t*-MA-U has an elimination half-life of five hours and reflects a short-term exposure to benzene [34].

Composite exposure indicator

In order to accurately represent the level of traffic exposure, we modeled it with a composite indicator that explained both the variability of the proxy-biomarker and the amount of contact with traffic. We estimated the amount of contact with traffic preceding the examination by:

$$t_i + log(DWTD_i),$$

where t_i is the standardized amount of time in traffic at the day of the examination and log*(DWTD$_i$)* is the standardized logarithm of the DWTD for person *i*. We standardized the amount of time in traffic and the logarithm of the DWTD in order to express them on the same scale. The standardization was achieved by centering and dividing by the standard deviation, i.e., $X_{std} = (X - \bar{X})/\sigma_X$. We used the DWTD for the location of the school when the examination took place from Monday to Friday (N=579), and for the residential location when the examination took place on Saturday (N=27). In terms of structural equation modeling, both time in traffic and traffic density are formative indicators, *i.e*. indicators that affect the level of traffic exposure. The use of formative indicators in structural equation modeling involves several problems concerning the identifiability of the model and the meaning of the construct they measure [35,36]. In order to deal with these problems, similarly to Treiblmaier et al. [36], we chose the weights describing the relative importance of the formative indicators (time in traffic and DWTD) prior to the analysis and specified the amount of contact with traffic the same way as reflective indicators (*i.e.*, indicators that are affected by the construct they measure). Commute exposure is responsible for a substantial fraction of the overall exposure to air pollution [37,38]. Since the commute to the schools preceded the examination, we considered time spent in traffic as an important contributor to traffic exposure. On the other hand, the relevance of spatial indicators of air pollution

exposure has been supported by multiple epidemiological studies, in which negative associations with performance in neurobehavioral tests have been observed [15,16,19-21,39,40]. Because no clear evidence was available suggesting that commute exposure contributed to the overall exposure more than traffic density or the opposite, we assigned the same weights to both sources of traffic exposure.

Neurobehavioral outcomes

We used the computerized battery of tests Neurobehavioral Evaluation System (NES3), which was developed to study neurobehavioral effects of exposure to environmental agents [41,42]. A detailed description of the specific tests that we administered can be found elsewhere [27]. Briefly, in the Continuous Performance Test, a series of 48 letters was presented on the screen. The task was to react as fast as possible to the letter S but not to other letters. We used the mean reaction time and the number of errors of omission as indicators of sustained attention. In the Digit Span Test, the task was to reproduce a series of digits after an auditory presentation. The maximum number of digits reproduced in the order of presentation (Digit Span Forward) and the maximum number of digits reproduced in the reverse order (Digit Span Backward) evaluated the short-term memory. The Finger Tapping Test required key tapping with the index finger. The total number of taps in four trials (10 sec each) with the preferred and non-preferred hand were used as indicators of manual motor speed.

Statistical analysis

In contrast to the classical regression methods, a structural equation model can include several indicators of one factor in a model [43]. This allows to reduce the measurement error and to represent the exposure, health outcomes, and confounders more adequately in the model. Moreover, structural equation modeling is not prone to the problem of multiple testing because a complex model including several indicators of the health outcome and exposure can investigate a hypothesis of interest with one statistical test. As main analysis, we fitted a structural equation model for each neurobehavioral domain (sustained attention, short-term memory, and manual motor speed) to study the association with the composite indicator of traffic exposure (Figure 2).

Gender, age, smoking, passive smoking, level of education of the mother, socioeconomic status of the family, time of the day, and the day of the week were *a priori* identified as potential confounders of the association between traffic exposure and neurobehavioral performance. We measured the socioeconomic status of the family with two indicators: the family income *per capita* and the highest occupational category of either parent. For all categorical

covariates, indicator variables were used (for the levels of the categorical covariates, see Table 1). Subjects who smoked during three days preceding the examination were considered smokers. For passive smoking we used a categorical variable indicating whether at least one family member smoked inside the home.

The parameters of the model were estimated using MCMC methods [44]. We applied a Bayesian approach because it allows to fit complex structural equation models when the sample size is not very large and to model non-continuous indicators and missing data in a straightforward way [43]. In order to handle non-continuous indicators (number of errors of omission, digit span forward, digit span backward, and occupation category), we treated them as coming from a hidden normal distribution [43,45]. In all analyses, both complete observations and observations with missing data were used. In each iteration of the Gibbs sampler, the missing values were sampled from the appropriate distributions. For covariates with missing values (smoking, passive smoking, and level of education of the mother), we applied a multivariate probit approach [46] and modeled the mean of the underlying normal distribution as a function of the socioeconomic status.

A Bayesian analysis requires a specification of the prior distributions of the parameters, which describe the knowledge before the analysis of the current data [47]. We used non-informative priors to let the information from the data dominate the inference. For the regression coefficients, we declared a prior distribution N(0;10,000). For the variance parameters, we specified a uniform prior on the standard deviations. Point estimation was based on the mean of the posterior distribution. For interval estimation, we used 95% Bayesian equal-tail credible intervals (95% BCI) [47]. We used the posterior distribution to calculate the posterior probability that there was a negative association between traffic exposure and the neurobehavioral domains. When this probability exceeded 95%, we concluded that strong evidence of a negative association was present. We used SAS (version 9.3) for data management and Winbugs (version 1.4) to fit the models. In all analyses, convergence was reached during the first 20,000 iterations. The parameter estimates were based on the following 10,000 iterations. The Winbugs code is available at request from the authors.

Additionally, we performed a secondary analysis. For each neurobehavioral domain, we fitted a model in which the exposure was indicated only by the level of *t,t*-MA-U and a model including all indicators of contact with traffic (school DWTD, residential DWTD, and time in traffic at the day of the examination). Additionally, we investigated the associations between the composite traffic exposure indicator and individual neurobehavioral parameters.

We considered smoking and passive smoking as possible sources of residual confounding. Therefore, we performed a sensitivity analysis, in which we excluded smokers and passive smokers. Additionally, we included the logarithm of the blood lead concentration as a covariate in the model in order to

investigate whether the effect of traffic exposure was independent of lead exposure. We also investigated whether the results were robust against a potential confounding of study area by including this variable in the models. Because *t,t*-MA-U is an indicator of a short-term traffic exposure, a long period between the collection of a urine sample and the neurobehavioral examination could introduce exposure misclassification, leading to an underestimation of the strength of the associations between traffic exposure and the neurobehavioral outcomes. Therefore, we performed a sensitivity analysis, in which we included only adolescents for whom the urine sample was collected not longer than four hours before the neurobehavioral examination (85% of the study group).

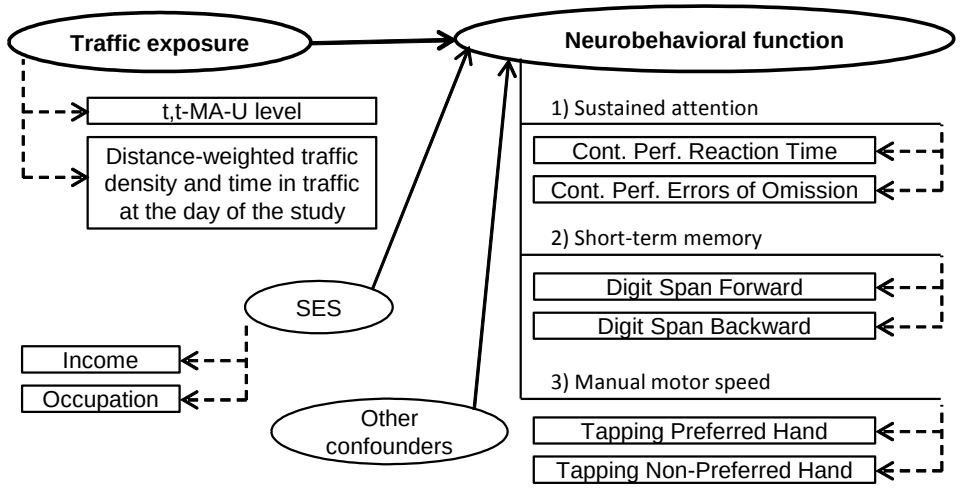

FIGURE 2. Overview of the main analyses.

RESULTS

Characteristics of the study group

Our study included 324 boys and 282 girls between 13.6 and 17.0 years of age. Table 1 shows characteristics of the participants and information about the average results in the neurobehavioral tests. The mother of 45% of the adolescents had a higher education degree. Characteristics of the distribution of the traffic exposure indicators are given in Table 2. For most variables, information for all or almost all participants was available (Table 1 and Table 2). The percentage of missing values was always smaller than 12%. Descriptive statistics for each study group separately (adolescents from Genk, Menen, and the general population) are given in Appendix A.

TABLE 1. Descriptive statistics.

N=606	
Demographic characteristics, N=606[a]	
Boys	324 (53.5%)
Age, years	14.9 (0.7)
Level of education of the mother, N=550[a]	
No diploma	38 (6.9%)
9 grades	72 (13.1%)
12 grades	190 (34.5%)
College or university diploma	250 (45.5%)
Highest category of occupation of either parents, N=559[a]	
Unemployed or not qualified worker	44 (7.9%)
Qualified worker, white-collar assistant or teaching staff	267 (47.8%)
Self-employed, specialist or member of management	248 (44.4%)
Household income *per capita* (Euro), N=535[a]	750 (296)
Smoking	
Smoking during three days preceding the examination, N=605[a]	34 (5.6%)
Passive smoking, N=598[a]	105 (17.6%)
Blood lead level (µg/dL), N=606[a]	1.51 (0.77)
Examination day, N=606[a]	
Monday	149 (24.6%)
Tuesday	254 (41.9%)
Thursday	166 (27.4%)
Friday	10 (1.7%)
Saturday	27 (4.5%)
Examination time, N=606[a]	
Before 10 am	224 (37%)
Between 10 am and noon	354 (58.4%)
Between noon and 2 pm	28 (4.6%)
Neurobehavioral parameters	
Continuous Performance (reaction time, msec), N=535[a]	410 (42)
Continuous Performance (number of errors of omission), N=535[a]	2.3 (2.67)
Digit Span Forward (number of digits), N=597[a]	5.57 (1.04)
Digit Span Backward (number of digits), N=579[a]	4.48 (1.03)
Finger Tapping, preferred hand (number of taps), N=597[a]	294 (42)
Finger Tapping, non-preferred hand (number of taps), N=595[a]	259 (35)

Arithmetic mean (standard deviation) is given for the continuous variables. Count (percent) is given for the categorical variables.
[a] Number of participants for whom information was available.

TABLE 2. Descriptive statistics for traffic exposure indicators.

	N	Mean	P5	P25	P50	P75	P95
***t,t*-MA-U, µg/g creat.**	605	105.3	18.3	32.1	58.9	124.4	358.0
Time in traffic, min/day	575	27.7	0.0	0.0	15.0	40.0	100
DWTD, km/day							
Residential	606	245	52	92	133	200	844
School location	601	232	13	91	128	392	655

t,t-MA-U: *trans,trans*-muconic acid in urine.
DWTD: distance-weighted traffic density.

Main analysis

The correlation between the amount of contact with traffic (based on traffic density and time spent in traffic) and log(*t,t*-MA-U) was 0.09 (95% CI: 0.01 to 0.17). The model for sustained attention explained 34.8% of the variability in this neurobehavioral domain. The other models explained 14.0%

and 7.9% of the variability in short-term memory and manual motor speed, respectively. Age, gender, and level of education of the mother were the most important predictors of neurobehavioral performance (Table 3).

TABLE 3. Parameter estimates in the main models.

	Sustained attention		Short-term memory		Manual motor speed	
	Effect size	**95% BCI**	**Effect size**	**95% BCI**	**Effect size**	**95% BCI**
Boys	-0.28	-0.55 to -0.02	-0.03	-0.26 to 0.21	0.36	0.16 to 0.56
Age, +1 year	0.38	0.18 to 0.58	0.00	-0.17 to 0.17	0.07	-0.07 to 0.21
Mother's education[a]						
no diploma	-0.70	-1.36 to -0.04	-0.66	-1.25 to -0.06	-0.22	-0.72 to 0.32
9 grades	-0.01	-0.47 to 0.44	-0.42	-0.84 to -0.01	0.14	-0.21 to 0.49
12 grades	-0.34	-0.66 to -0.02	-0.47	-0.77 to -0.17	0.16	-0.08 to 0.41
SES, +1 SD	0.04	-0.16 to 0.25	0.06	-0.13 to 0.24	0.17	0.01 to 0.33
Smokers	-0.36	-0.93 to 0.22	-0.06	-0.55 to 0.45	0.04	-0.37 to 0.44
Passive smokers	0.03	-0.32 to 0.40	0.03	-0.28 to 0.34	-0.02	-0.28 to 0.22
Day of the week[b]						
Tuesday	0.45	0.11 to 0.78	0.23	-0.06 to 0.52	0.08	-0.16 to 0.31
Thursday	0.06	-0.31 to 0.43	-0.02	-0.33 to 0.30	-0.08	-0.33 to 0.16
Friday	-0.01	-1.00 to 0.96	-0.21	-1.06 to 0.65	-0.07	-0.78 to 0.65
Saturday	-0.86	-1.58 to -0.17	-0.36	-0.94 to 0.21	-0.35	-0.81 to 0.12
Time[c]						
Before 10 am	-0.86	-1.54 to -0.18	-0.40	-1.00 to 0.16	-0.47	-0.97 to 0.09
10am – 12 am	-0.67	-1.31 to -0.03	-0.37	-0.94 to 0.17	-0.49	-0.97 to 0.04
Traffic, +1 SD	-0.26	-0.51 to -0.02	-0.17	-0.38 to 0.04	-0.10	-0.27 to 0.07

BCI: Bayesian credible interval.
The table shows the estimated number of standard deviation (SD) change in the neurobehavioral domains. Negative coefficients indicate a negative association of predictor with neurobehavioral performance.
[a] Reference category: college or university diploma.
[b] Reference category: Monday.
[c] Reference category: after 12 am.

Figure 3 depicts the posterior distribution of the regression coefficients describing the associations between traffic exposure (composite indicator) and the neurobehavioral domains. The coefficients express the number of standard deviations (SD) change in the neurobehavioral domains per one SD increase in traffic exposure. The posterior distributions describe the knowledge about the coefficients given the prior knowledge (no prior knowledge was assumed) and the data from the current study. The area under the curve for a certain range of values on the x-axis is the posterior probability that the regression coefficient takes a value within that range. For all neurobehavioral domains, there was a large posterior probability that the coefficients were smaller than 0 (P=98.3% for sustained attention, P=94.5% for short-term memory, and P=87.9% for manual motor speed), indicating a large posterior probability of a negative association between traffic exposure and the neurobehavioral domains. A one SD increase in the exposure was associated with a 0.26 SD decrease in sustained attention (95% BCI: -0.51 to -0.02, Table 4). The associations between traffic exposure and the remaining neurobehavioral domains had the same direction but the credible intervals included zero (Table 4).

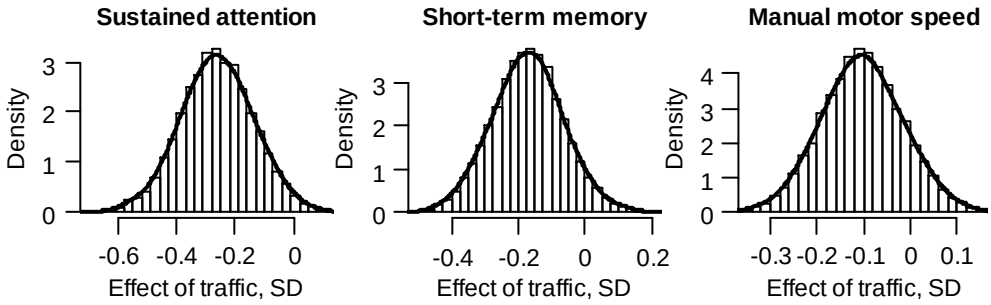

FIGURE 3. Posterior distribution of the regression coefficients describing the change in neurobehavioral domains associated with a one SD increase in traffic exposure.

The area under the curve for a certain range of values on the x-axis is the posterior probability that the regression coefficient takes a value within that range.

Secondary analysis

In the secondary analysis, we explored the associations between the individual indicators of traffic exposure and the three neurobehavioral domains (Table 4). A two-fold increase in the level of *t,t*-MA-U was associated with a 0.11 SD decrease in sustained attention (95% BCI: -0.20 to -0.02). For the two remaining neurobehavioral domains, the point estimates of the regression coefficients were consistent but the credible intervals included zero. We did not find evidence that DWTD or time in traffic alone were significantly associated with neurobehavioral performance (Table 4).

Additionally, we investigated the associations between the individual neurobehavioral parameters and the composite indicator of traffic exposure. A one SD increase in the exposure was associated with an 8.76 msec longer reaction time (95% BCI: 1.86 to 15.7) and 0.17 SD shorter digit span backward (-0.34 to -0.01). For the other neurobehavioral parameters, the point estimates of the regression coefficients were consistent but the credible intervals included zero (Table 5).

TABLE 4. Associations between traffic exposure indicators and neurobehavioral domains.

	Sustained attention			Short-term memory			Manual motor speed		
	Effect size	**95% BCI**	**P(β<0)**	**Effect size**	**95% BCI**	**P(β<0)**	**Effect size**	**95% BCI**	**P(β<0)**
Main analysis									
Composite indicator, +1SD	-0.26	-0.51 to -0.02	98.3%	-0.17	-0.38 to 0.04	94.5%	-0.10	-0.27 to 0.07	87.9%
Secondary analysis, model 1									
t,t-MA-U, ×2	-0.11	-0.20 to -0.02	99.1%	-0.06	-0.13 to 0.01	94.2%	-0.03	-0.10 to 0.03	83.2%
Secondary analysis, model 2									
Residential DWTD, ×2	-0.02	-0.12 to 0.08	62.2%	0.07	-0.01 to 0.16	4.9%	0.05	-0.02 to 0.13	6.7%
School DWTD, ×2	-0.05	-0.14 to 0.04	87.5%	-0.01	-0.08 to 0.07	55.7%	-0.01	-0.07 to 0.06	57.6%
Time in traffic, +1h	-0.06	-0.29 to 0.16	69.2%	-0.03	-0.22 to 0.17	60.0%	-0.08	-0.24 to 0.07	85.0%

BCI: Bayesian credible interval; P(β<0): the posterior probability of a negative association; *t,t*-MA-U: *trans,trans*-muconic acid in urine; DWTD: distance-weighted traffic density.

The table shows the number of standard deviations change in the neurobehavioral domains. For the composite exposure indicator, the effect of a one standard deviation increase is given. For *t,t*-MA-U and the DWTD, the effect of a two-fold increase is given. For time in traffic, the effect of a 1 hour increase is given. The model took into account gender, age, smoking, passive smoking, level of education of the mother, socioeconomic status, time of the day, and day of the week.

TABLE 5. Associations between the composite indicator of traffic exposure and neurobehavioral parameters.

	Effect size	95% BCI	P
Continuous Performance			
Mean reaction time (msec)	8.76	1.86 to 15.7	99.3%[a]
Errors of omission (SD)	0.07	-0.10 to 0.24	78.5%[a]
Digit Span			
Maximum span forward (SD)	-0.06	-0.22 to 0.10	75.8%[b]
Maximum span backward (SD)	-0.17	-0.34 to -0.01	98.0%[b]
Finger Tapping (number of taps)			
Preferred hand	-3.98	-10.4 to 2.47	88.5%[b]
Non-preferred hand	-2.72	-7.98 to 2.56	84.5%[b]

[a] P(β>0): the posterior probability of a positive association.
[b] P(β<0): the posterior probability of a negative association.
BCI: Bayesian credible interval.
The effect of a one standard deviation (SD) increase of the exposure is given. For non-continuous parameters (errors of omission, maximum span forward and backward) a probit model was used and the effects are expressed as the number of standard deviations. The model took into account gender, age, smoking, passive smoking, level of education of the mother, socioeconomic status, time of the day, and day of the week.

Sensitivity analysis

In the analysis excluding smokers and passive smokers, a one SD increase in traffic exposure was associated with a 0.28 SD decrease in sustained attention (95% BCI: -0.55 to -0.01) and 0.11 SD decrease in manual motor speed (95% BCI: -0.29 to 0.09). Hence, the sensitivity analysis suggested that residual confounding of smoking or passive smoking did not affect the estimates of the association between traffic exposure and sustained attention or manual motor speed. However, the estimate of the size of the association between traffic exposure and short-term memory was smaller in the analysis excluding smokers and passive smokers (0.12 SD decrease for a 1 SD increase in the exposure, 95% BCI: -0.35 to 0.10) than in the main analysis.

Including the logarithm of the blood lead level had little influence on the associations between traffic exposure and the neurobehavioral domains. According to the model accounting for the blood lead level, a one SD increase in traffic exposure was associated with a 0.25 SD decrease in sustained attention (95% BCI: -0.49 to -0.01). Including study area in the models did not change the estimates more than by 0.01. In the analysis including only adolescents for whom a urine sample was collected within four hours before the neurobehavioral examination (i.e., 85 % of the total study group), a one SD increase in traffic exposure was associated with a 0.27 SD decrease in sustained attention (95% BCI: -0.54 to 0.00).

DISCUSSION

Our study in a group of 606 adolescents showed an inverse association between sustained attention and traffic exposure. The sustained attention score decreased by 0.26 SD for a one SD increase in the composite exposure indicator, which we constructed using a proxy-biomarker of traffic exposure (the level of *t,t*-MA-U) and the estimate of the amount of contact with traffic preceding the neurobehavioral examination (based on traffic density and time spent in traffic). Although this mild association does not seem very important in terms of the amount of distress caused to individuals, it may have serious public health implications on a population level [48]. The public health significance of a decrease in sustained attention by 0.26 SD can be illustrated by the fact that it corresponded to one-third of the estimated difference between children of a mother without any diploma and with a university or college diploma. The inverse association between sustained attention and traffic exposure was independent of the blood lead level and potential confounders including the level of education of the mother, the socioeconomic status, smoking, and passive smoking. The associations with short-term memory and manual motor speed had the same direction but were not statistically significant.

The inverse association between traffic exposure and sustained attention in a population of Flemish adolescents is in line with the findings from two recent studies in children [16,40]. A study in Boston evaluated the traffic exposure using residential black carbon concentrations and administered neurobehavioral tests in a group of 174 children between 7 and 14 years of age. The black carbon level showed a strong negative association with sustained attention measured by the Continuous Performance Test [16]. A Chinese study evaluated the neurobehavioral function in children attending two schools with a different level of air pollution. Children whose school was located in an area with a low traffic density performed better in the Continuous Performance Test than those from a school with much traffic-related air pollution [40]. In elderly, the residential distance to a busy road was inversely associated with selective attention measured by the Stroop Test [19].

The secondary analysis showed that a one SD increase in traffic exposure was associated with an 8.76msec (95% BCI: 1.86 to 15.7) longer mean reaction time in the Continuous Performance Test. For the number of errors of omission, the second indicator of sustained attention, the association with traffic exposure was not statistically significant. In contrast to the number of errors of omission, which is mainly a measure of inattention, the reaction time involves speed in processing of visual information. This higher-order cognitive skill may be more vulnerable to environmental insults than the ability not to get distracted [49].

In children between 9 and 11 years of age, the average annual nitrogen dioxide concentrations at locations of the schools were negatively associated with the average number of digits reproduced in the forward and backward Digit Span Test [50]. In elderly women, residential particulate matter concentrations were associated with an increase in the rate of performance decline in the Digit Span Test backward [21]. Consistent with these results, our study showed a high posterior probability of a negative association between traffic exposure and short-term memory. However, excluding smokers and passive smokers in the sensitivity analysis led to a decrease in the estimate of the size of the association, suggesting a possibility of residual confounding of these two factors.

The composite indicator of traffic exposure included the level of *t,t*-MA-U, a metabolite of benzene. Benzene, a well-known hematotoxic and carcinogenic agent [51], may also affect the brain. In rodents, benzene is capable of inducing lethargy [52], hyperactivity [53], and changes in the level of monoamine neurotransmitters [54]. In humans, symptoms such as headache, dizziness, and drowsiness have been reported after an acute benzene exposure [55,56]. With an elimination half-life of five hours *t,t*-MA-U mostly reflects benzene exposure during the past few hours [34]. The exposure to benzene from traffic exhaust is accompanied by other toxicants including nitrogen oxides, carbon monoxide, polycyclic aromatic hydrocarbons, toxic metals such as lead and manganese, and noise. Therefore, we considered the level of *t,t*-MA-U as a proxy-biomarker for traffic, not a mere biomarker of benzene exposure. The sensitivity of *t,t*-MA-U as an indicator of traffic-related air pollution exposure has been supported by studies showing that the *t,t*-MA-U level strongly increases as a result of exposure to traffic exhaust [23-25]. Cigarette smoke [57] and sorbic-acid preserved foods [58] may also affect the level of *t,t*-MA-U. Therefore, to ensure the specificity of the traffic indicator, we used structural equation modeling to create a composite indicator that explained both the variability in *t,t*-MA-U and the amount of contact with traffic (indicated by traffic density and time spent in traffic).

There are several pathways by which traffic exposure may affect the neurobehavioral function. Airborne fine particles enter the brain via the olfactory nerve or the blood-brain barrier and deposit in different regions of the brain, which may induce neuroinflammation [2,3,14]. Besides this direct pathway, air pollution causes a pro-inflammatory response in the cardiovascular and respiratory systems and the liver, leading to increased systemic inflammation [59,60], which in turn, can induce inflammatory changes in the brain [8]. Increased neuroinflammation may affect neurotrophins, such as the brain-derived neurotrophic factor, which regulates the survival, differentiation and phenotypic maintenance of various neuronal populations. Both animal [61] and intervention studies in humans [62] have shown decreases in the expression of brain-derived neurotrophic factor in response to air pollution.

Additionally, the cardiovascular changes caused by air pollution may be responsible for the neurobehavioral effects. Recently, we observed in a panel of healthy subjects immediate responses of the retinal microcirculation associated with particulate matter and black carbon [63]. The vascular network of the brain, as exemplified by the retinal blood vessels, may be crucial for cognitive ability as it supplies oxygen and nutrients. Finally, traffic noise may be an important contributor to the negative effects related to traffic exposure, for example by affecting the sleep quality [50,64].

The main limitation of our study is its cross-sectional design. We cannot exclude that some covariates were not adequately represented in the models leading to residual confounding. However, in order to deal with this problem, we took several precautions. First, our models accounted for a large number of potential confounders, including socioeconomic status, smoking and passive smoking. Second, we ascertained the quality of the assessment of the socioeconomic status by using two indicators (the family income *per capita* and the highest occupational category of either parent). Third, we performed a sensitivity analysis that excluded smokers and passive smokers. Although our study alone does not allow to draw conclusions about causality, it adds to a growing body of evidence from epidemiological and experimental animal research suggesting that traffic exposure may affect the neurobehavioral function. Another limitation of our study is that the urine sample was not collected at the moment of the neurobehavioral examination. This could introduce misclassification of the exposure and, consequently, lead to an underestimation of the associations between traffic exposure and neurobehavioral performance. However, the estimates from the sensitivity analysis using only adolescents for whom a urine sample was collected not longer than four hours before the neurobehavioral examination (85 % of the study group) were similar to those based on the whole study group.

Our study has several strengths. We gathered different sources of information about the traffic exposure including the level of *t,t*-MA-U, traffic density, and information about time spent in traffic. The application of structural equation modeling allowed us to combine them in one model. By creating a factor that explained both the variability in *t,t*-MA-U and in the estimate of the exposure based on traffic density and time spent in traffic, we increased the chance that the exposure was accurately represented in the model. Furthermore, to improve the quality of the evaluation of the neurobehavioral domains, we measured each of them with two indicators. Last but not least, our main analysis did not suffer from the problem of multiple testing because we investigated each hypothesis with one statistical test.

CONCLUSIONS

Our study in a population of adolescents supports the recent findings in children and elderly suggesting that neurobehavioral performance may be negatively associated with traffic exposure. The comparison between the magnitude of decline in attention associated with traffic exposure and with a lower level of maternal education suggests that traffic exposure may substantially decrease attention in adolescents, potentially affecting learning, problem solving, and school performance in general.

APPENDIX A. CHARACTERISTICS OF DIFFERENT STUDY GROUPS

	All participants, N=606	General population, N=210	Genk, N=197	Menen, N=199	p-value
Demographic characteristics, N=606[a]					
Boys	324 (53.5%)	121 (57.6%)	89 (45.2%)	114 (57.3%)	0.018[b]
Age, years	14.9 (0.7)	14.8 (0.5)	15 (0.7)	15.1 (0.8)	<.001[c]
Level of education of the mother, N=550[a]					<.001[b]
No diploma	38 (6.9%)	4 (2%)	26 (15.1%)	8 (4.4%)	
9 grades	72 (13.1%)	25 (12.7%)	21 (12.2%)	26 (14.4%)	
12 grades	190 (34.5%)	77 (39.1%)	51 (29.7%)	62 (34.3%)	
College or university diploma	250 (45.5%)	91 (46.2%)	74 (43%)	85 (47%)	
Highest category of occupation of either parents, N=559[a]					0.008[b]
Unemployed or not qualified worker	44 (7.9%)	8 (4%)	22 (12.6%)	14 (7.7%)	
Qualified worker, white-collar assistant or teaching staff	267 (47.8%)	95 (47.3%)	90 (51.4%)	82 (44.8%)	
Self-employed, specialist or member of management	248 (44.4%)	98 (48.8%)	63 (36%)	87 (47.5%)	
Income *per capita* (euro), N=535[a]	750 (296)	781 (297)	686 (284)	785 (297)	0.001[c]
Smoking					
Active during previous 3 days, N=605[a]	34 (5.6%)	15 (7.2%)	7 (3.6%)	12 (6%)	0.272[b]
Passive, N=598[a]	105 (17.6%)	37 (18%)	35 (17.9%)	33 (16.7%)	0.922[b]
Blood lead level (µg/dL), N=606[a]	1.51 (0.77)	1.64 (0.96)	1.50 (0.73)	1.38 (0.53)	0.004[c]
Examination day, N=606[a]					<.001[b]
Monday	149 (24.6%)	19 (9%)	109 (55.3%)	21 (10.6%)	
Tuesday	254 (41.9%)	169 (80.5%)		85 (42.7%)	
Thursday	166 (27.4%)	12 (5.7%)	71 (36%)	83 (41.7%)	
Friday	10 (1.7%)	10 (4.8%)			
Saturday	27 (4.5%)		17 (8.6%)	10 (5%)	
Examination time, N=606[a]					0.246[b]
Before 10 am	224 (37%)	83 (39.5%)	67 (34%)	74 (37.2%)	
Between 10 am and noon	354 (58.4%)	120 (57.1%)	123 (62.4%)	111 (55.8%)	
Between noon and 2 pm	28 (4.6%)	7 (3.3%)	7 (3.6%)	14 (7%)	
Traffic exposure					
t,t-MA-U, µg/g creat, N=605[a]	105 (135)	105.3 (140)	115 (157)	95 (105)	0.589[c]
Time in traffic, min/day, N=575[a]	27.7 (35.6)	30 (36.1)	38.1 (38.6)	14.9 (27.2)	<.001[d]
Residential DWTD, km/day, N=606[a]	245 (353)	241 (479)	210 (240)	284 (281)	<.001[c]
School DWTD, km/day, N=601[a]	232 (251)	253 (295)	168 (250)	273 (180)	<.001[d]

Arithmetic mean (standard deviation) is given for continuous variables. Count (percent) is given for the categorical variables.

[a] Number of participants for whom information was available.

[b] Pearson Chi-square test.

[c] ANOVA, for blood lead level, *t,t*-MA-U, and DWTD log-transformed variables were used.

[d] Kruskal-Wallis ANOVA.

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Chapter 5

Neurobehavioral changes in adolescents and urinary *t,t*-muconic acid used as proxy-biomarker of traffic exposure

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ABSTRACT

Introduction: Traffic-related air pollution induces neurotoxicity in rodents. Several recent epidemiological studies reported negative associations between residential exposure and neurobehavioral performance. Here, we investigated in a population of adolescents the associations between the urinary concentration of *trans,trans*-muconic acid (*t,t*-MA-U), a metabolite of benzene used as proxy-biomarker of traffic exposure, and two neurobehavioral domains: sustained attention and short-term memory. Additionally, we investigated whether these associations were modified by the education level of the parents or family income.

Methods: As a part of a biomonitoring program for environmental health surveillance in Flanders (Belgium), we examined between 2008 and 2014 grade nine high school students. An HPLC-based method was used to determine *t,t*-MA-U. We used the mean reaction time and the number of omission errors in the Continuous Performance Test to measure sustained attention and digit span forward and digit span backward to measure short-term memory. All estimates were adjusted for gender, age, passive smoking, the education level of the parents, family income, time of the day, day of the week, and the logarithm of blood lead level.

Results: A total of 931 non-smoking adolescents underwent the neurobehavioral examination. A ten-fold increase in *t,t*-MA-U was associated with a 0.17 SD decrease in sustained attention (95% Confidence Interval: -0.31 to -0.04) and a 0.13 SD decrease in short-term memory (95% CI: -0.28 to 0.01). We found no evidence that these associations were modified by the education level of the parents or family income.

Conclusions: The concentration of *t,t*-MA-U was associated with a decreased sustained attention in adolescents.

INTRODUCTION

Historically, traffic was considered a threat to children's mental health because of its contribution to environmental lead contamination [1]. Despite the phase-out of lead, a number of recent studies reported negative associations between neurobehavioral outcomes and indicators of traffic-related air pollution exposure [2-6]. These observations from epidemiological research have been supported by experimental studies showing that traffic-related air pollution induces neurobehavioral effects in rodents [7-9].

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 an internal biomarker of 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, polycyclic aromatic hydrocarbons, toxic metals, and benzene, a constituent of gasoline. *Trans,trans*-muconic acid (*t,t*-MA), a urinary metabolite of benzene, is a useful proxy-biomarker of inhalation exposure from traffic integrating exposures at different locations and the commute-related exposure as demonstrated by several studies showing that the urinary concentration of *t,t*-MA (*t,t*-MA-U) strongly increases as a result of exposure to traffic exhausts [10-12]. Furthermore, the usefulness of *t,t*-MA-U as a biomarker of traffic exposure in Flemish adolescents has been supported by a study, which showed 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 [13].

Disadvantaged populations have a less healthy and comfortable lifestyle and a poorer health status compared to people with a high socioeconomic status [14]. As environmental pollution may act in synergy with these factors, populations with a low socioeconomic status may be more susceptible to the health effects of environmental pollutants than those with a high socioeconomic status [15,16]. The effect modification of socioeconomic indicators on the association between traffic exposure and neurobehavioral performance has not been studied to date.

Here, we investigated in Flemish adolescents the association between two neurobehavioral domains (sustained attention and short-term memory) and traffic exposure as indicated by *t,t*-MA-U. Additionally, we studied the effect modification by socioeconomic status.

METHODS

Study population and data collection

The study was a part of a biomonitoring program for environmental health surveillance in Flanders, Belgium. Due to the high population density, the popularity of cars as means of transport, the low frequency of car-pooling, and the presence of the urban sprawl requiring the inhabitants to travel frequently and relatively far [17], the concentrations of traffic-related air pollutants in Flanders exceed the World Health Organization (WHO) norms. For example, the mean annual concentrations of $PM_{2.5}$ exceeded year after year the WHO guideline of 10 μg/m^3 for all measuring stations (both rural and urban) [18-20].

Between 2008 and 2014, we invited grade nine high school students. Most of them were 14 or 15 years of age and only those who spoke Dutch were eligible. A part of 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 proportional to the number of inhabitants in that province. The other part of the study group was recruited in the municipalities of Genk, Menen, and the port area of Ghent. In this case, addresses of adolescents were obtained from the population registry 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 examination, subjects received two questionnaires to fill out, one for themselves and one for their parents. The questionnaire for the adolescents included information about their smoking behavior. 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 [21]. Questions about the socioeconomic status and passive smoking were included in the questionnaire for the parents. Family income was standardized by the number of family members by dividing the total income by $0.5+0.3\times n_c+0.5\times n_a$, where n_c is the number of family members younger than 18 years of age and n_a is the number of family members above 18 years of age [22]. As indicator of the education level of the parents, we used the highest education level of either parent. Adolescents exposed to secondary tobacco smoke at home were classified as passive smokers.

A day before the examination, each participant received a plastic bottle and was asked to collect a first morning urine sample at the day of the neurobehavioral examination. The urine samples were brought to the schools,

stored in a cooler (4°C), and kept frozen at -20°C until analysis. Both parents and teenagers provided informed consent for participation. The study was approved by the Ethical Committee of the University of Antwerp.

Biological measurements

We measured the concentrations of *t,t*-MA-U using the method of Angerer et al. [23]. 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 were measured using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) [24].

Neurobehavioral outcomes

The Neurobehavioral Evaluation System (NES) is a computerized battery of tests that was developed to study the neurobehavioral effects of neurotoxicants. 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) [25,26].

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 was displayed each 1000 msec and remained on the screen for 200 msec. The test consisted of five blocks of 12 letters and the last four blocks were used to compute the performance indicators. To measure sustained attention, we used the mean reaction time for correct responses and the number of errors of omission (i.e. the number of non-responses).

In the Digit Span Test, the task was to reproduce sequences of digits after an auditory presentation. The test consisted of a forward and a backward part. The forward part of the test started with a sequence of three digits. When a sequence was correctly reproduced, a new sequence was presented that consisted of one digit more than the previous one. Otherwise, a sequence of the same length was presented. The forward part of the test stopped when a subject failed to reproduce two sequences in a row. In the backward condition, the task was to reproduce digits in the reverse order. The maximum numbers of digits correctly reproduced in the order of the presentation (digit span forward) and in the reverse order (digit span backward) were used to measure short-term memory.

Statistical analysis

We transformed *t,t*-MA-U and blood lead level logarithmically to reduce the skewness of their distributions. In the main analysis, we investigated the association between *t,t*-MA-U and sustained attention using a bivariate latent variable model for a clustered continuous (the mean reaction time in the Continuous Performance Test) and discrete (the number of errors of omission) outcome [27,28]. For short-term memory, we used a bivariate ordinal probit model [29]. Multiple imputation was applied to handle missing values [30]. We created 50 versions of the complete data based on an imputation model that included all variables used in the analysis. The Rubin's rules were used to estimate the regression coefficients [31].

As potential confounders, we considered gender, age, passive smoking, family income, the highest level of education of either parent, blood lead level, day of the week, and time of the day. All categorical covariates were modeled with indicator variables.

We investigated whether the strength of the association between *t,t*-MA-U and neurobehavioral performance was different for a low and high socioeconomic status by adding the interaction term to the models. We used two indicators of a low socioeconomic status: the lowest category of the education level and the lowest category of family income.

As secondary analysis, we investigated the associations between *t,t*-MA-U and individual neurobehavioral parameters, i.e., the mean reaction time and the number of errors of omission in the Continuous Performance Test, digit span forward, and digit span backward. In order to better understand the association between *t,t*-MA-U and the mean reaction time in the Continuous Performance Test, we additionally considered the evolution of the performance during the test. In this analysis, we used a mixed model with 5 responses (mean reaction times in five blocks of the test) and estimated the effect of exposure on the slope (i.e., the mean difference between two consecutive blocks). The model included the random intercept and slope and the same confounders as those taken into account in the main analysis. We used SAS software version 9.4 (SAS Institute Inc, Cary, NC) for all analyses.

RESULTS

Characteristics of the study group

The first column of Table 1 shows characteristics of the study group consisting of 931 non-smoking adolescents. The mean age was 14.9 years and 482 (51.8%) of the study participants were boys. For 13.4% of the adolescents,

none of the parents had a high school diploma. Family income standardized by the number of family members was lower than or equal to 1250 euro per month for 33.8% of the study participants. The geometric mean (range) of the concentrations of lead in the blood was 11.7 (2.7-76.9) µg/L. The geometric mean (range) of the concentrations of *t,t*-MA-U was 55.1 (2-1304) µg/g creatinine. The range of *t,t*-MA-U without standardization for creatinine concentration was 3 to 2008 µg/L. Table 1 also shows the participants' characteristics by *t,t*-MA-U tertiles. The distributions of the education level of the parents, family income, passive smoking, and day and time of the examination did not differ across the *t,t*-MA-U tertiles. Blood lead level was higher for higher *t,t*-MA-U concentrations. The number of missing values was smaller than 9% for all variables used in the analysis.

TABLE 1. Characteristics of all participants and by tertiles of *trans,trans*-muconic acid.

	All participants	*t,t*-MA-U tertiles			p-value
		1st tertile (2-34)[a]	2nd tertile (34-72)[a]	3rd tertile (72-1304)[a]	
Demographics, N=931[b]					
Boys	482 (51.8%)	165 (53.1%)	152 (49%)	165 (53.2%)	0.45[c]
Age (years)	14.89 (0.63)	14.86 (0.62)	14.93 (0.65)	14.87 (0.62)	0.57[d]
Education of parents, N=909[b]					0.09[c]
No high school diploma	122 (13.4%)	32 (10.5%)	47 (15.6%)	43 (14.2%)	
High school diploma	294 (32.3%)	91 (29.8%)	103 (34.1%)	100 (33.1%)	
College or university diploma	493 (54.2%)	182 (59.7%)	152 (50.3%)	159 (52.6%)	
Household income, N=849[b]					0.28[c]
≤1250 € / month	287 (33.8%)	95 (32.5%)	88 (31%)	104 (38.1%)	
>1250 - 1600 € / month	200 (23.6%)	68 (23.3%)	74 (26.1%)	58 (21.2%)	
>1600 - 2000 € / month	173 (20.4%)	64 (21.9%)	54 (19%)	55 (20.1%)	
>2000 € / month	189 (22.3%)	65 (22.3%)	68 (23.9%)	56 (20.5%)	
Passive smoking, N=917[b]	135 (14.7%)	47 (15.4%)	45 (14.8%)	43 (14%)	0.97[c]
Examination day, N=931[b]					0.63[c]
Monday	252 (27.1%)	91 (29.3%)	88 (28.4%)	73 (23.5%)	
Tuesday-Friday	632 (67.9%)	206 (66.2%)	205 (66.1%)	221 (71.3%)	
Saturday	47 (5%)	14 (4.5%)	17 (5.5%)	16 (5.2%)	
Examination time, N=931[b]					0.19[c]
Between 8 and 11 am	622 (66.8%)	195 (62.7%)	213 (68.7%)	214 (69%)	
Between 11 am and 13 am	230 (24.7%)	91 (29.3%)	72 (23.2%)	67 (21.6%)	
Between 13 and 16 pm	79 (8.5%)	25 (8%)	25 (8.1%)	29 (9.4%)	
Blood lead level (µg/L), N=931[b]	11.7 (1.6)	10.9 (1.5)	11.4 (1.6)	12.9 (1.5)	<0.001[d]
t,t-MA-U, µg/g creatinine, N=931[b]	55.1 (2.5)	22.6 (1.5)	48.3 (1.2)	153.7 (1.9)	<0.001[d]

Count (percent) is given for the categorical variables. Geometric mean (geometric standard deviation) is shown for blood lead and *trans,trans*-muconic acid in urine (*t,t*-MA-U). Arithmetic mean (standard deviation) is given for the remaining continuous variables.
[a] Range, µg/g creatinine.
[b] Number of participants for whom information was available.
[c] Pearson's Chi-square test.
[d] ANOVA.

Main analysis

The parents' education level was an important predictor of the two neurobehavioral domains studied (Table 2). Sustained attention increased with

age of the participants, was higher in girls than boys, and was higher from Tuesday to Friday in comparison to Saturday. None of the other variables had a significant effect on sustained attention or short-term memory.

TABLE 2. Estimates of the effects of covariates in the main models.

	Sustained attention		Short-term memory	
	Effect size	**95% CI**	**Effect size**	**95% CI**
Boys	-0.12	-0.23 to -0.00	0.05	-0.07 to 0.16
Age, +1 year	0.20	0.11 to 0.28	0.03	-0.05 to 0.12
Education of the parents[a]				
No high school diploma	-0.37	-0.55 to -0.18	-0.43	-0.63 to -0.24
High school diploma	-0.20	-0.33 to -0.07	-0.30	-0.44 to -0.17
Family income[b]				
≤1250 € / month	-0.02	-0.20 to 0.16	-0.12	-0.30 to 0.07
>1250 - 1600 € / month	0.04	-0.12 to 0.21	-0.16	-0.34 to 0.01
>1600 - 2000 € / month	-0.04	-0.21 to 0.13	-0.16	-0.33 to 0.02
Passive smoking	0.03	-0.13 to 0.19	0.02	-0.15 to 0.18
Examination day[c]				
Monday	0.20	-0.06 to 0.47	0.05	-0.22 to 0.32
Tuesday-Friday	0.32	0.07 to 0.57	0.21	-0.05 to 0.47
Examination time[d]				
Between 8am and 11am	0.01	-0.18 to 0.21	0.06	-0.15 to 0.26
Between 11am and 1pm	0.01	-0.20 to 0.21	0.06	-0.16 to 0.28
Blood lead level, ×2	-0.06	-0.15 to 0.03	-0.05	-0.15 to 0.04

CI: confidence interval.
The table shows the estimated number of standard deviation change in the neurobehavioral domains. Negative coefficients indicate a negative association of predictor with neurobehavioral performance.
[a] Reference category: college or university diploma.
[b] Reference category: >2000 € / month.
[c] Reference category: Saturday.
[d] Reference category: between 1pm and 4pm.

A ten-fold increase in *t,t*-MA-U was associated with a 0.17 SD decrease in sustained attention (95% Confidence Interval: -0.31 to -0.04) and a 0.13 SD decrease in short-term memory (95% CI: -0.28 to 0.01), correcting for gender, age, passive smoking, the education level of the parents, family income, time of the day, day of the week, and the logarithm of blood lead level (Table 3). We found no evidence of effect modification by the education level of the parents (p=0.40 for sustained attention, p=0.89 for short-term memory). The strength of the association between *t,t*-MA-U and sustained attention did not differ significantly between adolescents from families with a low income and other adolescents (p=0.51). Family income did not significantly modify the association between *t,t*-MA-U and short-term memory either (p=0.54).

TABLE 3. Associations between *trans,trans*-muconic acid in urine and neurobehavioral domains.

	Effect size	95% CI	p-value
Sustained attention	-0.17	-0.31 to -0.04	0.012
Short-term memory	-0.13	-0.28 to 0.01	0.069

CI: confidence interval.
The table shows the number of standard deviation change in the neurobehavioral domains for a ten-fold increase in traffic exposure as reflected by the level of trans,trans-muconic acid in urine. The mean reaction time and the number of errors of omission in the Continuous Performance Test were used to measure sustained attention. Maximum span forward and backward in the Digit Span Test were used to measure short-term memory. All models included gender, age, passive smoking, the education level of the parents, family income, time of the day, day of the week, and the logarithm of blood lead level.

Secondary analysis

A ten-fold increase in *t,t*-MA-U was associated with an 8.5 msec (95% CI: 1.4 to 15.7) slower mean reaction time in the Continuous Performance Test (Table 4, Figure 1). The model with the mean reaction times at 5 blocks as responses provided no evidence of an association between *t,t*-MA-U and the evolution of performance during the test (p=0.09). In other words, no differences in the association of *t,t*-MA-U with reaction time between different parts of the test were apparent. The association with the second parameter used to measure sustained attention, i.e., the number of errors of omission, was not statistically significant. The estimates of the association between *t,t*-MA-U and the parameters measuring short-term memory were almost the same for maximum span forward and backward and did not reach the statistical significance (Table 4).

TABLE 4. Associations between *trans,trans*-muconic acid in urine and neurobehavioral parameters.

	Effect size	95% CI	p-value
Continuous Performance Test			
Mean reaction time (msec)	8.54	1.41 to 15.66	0.019
Errors of omission (SD)	0.14	-0.04 to 0.33	0.120
Digit Span Test			
Maximum span forward (SD)	-0.13	-0.30 to 0.05	0.160
Maximum span backward (SD)	-0.14	-0.32 to 0.04	0.119

CI: confidence interval.
The effect of a ten-fold increase in traffic exposure as reflected by the level of *trans,trans*-muconic acid in urine is shown. For the mean reaction time in the Continuous Performance Test the effect is expressed in msec. For the remaining parameters a probit model was used and the effects are expressed as the number of standard deviations. All models included gender, age, passive smoking, the education level of the parents, family income, time of the day, day of the week, and the logarithm of blood lead level.

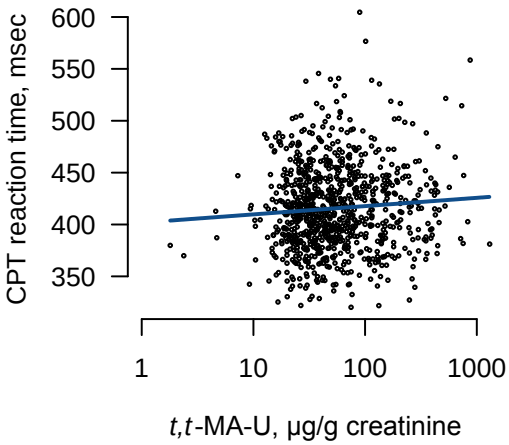

FIGURE 1. Scatter plot of the urinary levels of *trans,trans*-muconic acid (*t,t*-MA-U) and the mean reaction time in the Continuous Performance Test (CPT).

DISCUSSION

In a study including more than 900 non-smoking adolescents, we found evidence of an inverse association between a proxy-biomarker of traffic exposure, *t,t*-MA-U, and neurobehavioral performance. A ten-fold increase in the level of *t,t*-MA-U was associated with a 0.17 SD decrease in sustained attention (95% CI: -0.31 to -0.04) and a 0.13 SD decrease in short-term memory (95% CI: -0.28 to 0.01). We found no evidence that these associations were modified by the education level of the parents or family income.

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 [3] and sustained attention assessed by the Continuous Performance Test [6]. In a Chinese study of 928 nine years old children, those living in an area with high concentrations of ambient particulate matter and NO_2 showed poorer performance in a number of cognitive domains including sustained attention assessed by the Continuous Performance Test, compared to those living in a clean area [5]. Perinatal exposure to airborne polycyclic aromatic hydrocarbons (PAHs) measured with personal monitors was associated with a lower intelligence at age five [4,32]. In the same cohort, an inverse association between perinatal exposure to PAHs and the white matter surface has been

recently reported [33]. Observational human studies are supported by 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 [7]. Another study observed a negative effect of a long-term $PM_{2.5}$ exposure on discriminative memory [8]. Our observation of a negative association between *t,t*-MA-U and neurobehavioral performance in Flemish adolescents adds to the body of evidence suggesting that traffic-related air pollution exposure negatively affects cognition.

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 [34]. To prevent hematotoxic and carcinogenic health effects in exposed workers, the American Conference of Governmental Industrial Hygienists set the threshold limit value (TLV, time-weighted-average) for benzene at the stringent level of 0.5 ppm (1.6 mg/m³) in 1996 [35]. For comparison, the annual averages of benzene levels in 17 sites monitoring the quality of ambient air in Flanders ranged in 2011 from 0.53 to 1.97 µg/m³[18], which is more than 800 times lower than the TLV for the industrial environment. Currently, there is no evidence that such a low environmental exposure to benzene may cause neurotoxic effects. Benzene may account for 3 to 15 % of the total tailpipe hydrocarbon composition [34]. Vehicle exhaust is considered the largest anthropogenic source of environmental exposure to benzene, estimated to contribute for 70 to 80% to the overall man-made benzene emissions [34].

Because benzene-linked neurotoxic effects are not likely to occur as a result of traffic exposure and because exposure to traffic-related benzene is accompanied by exposure to other pollutants including nitrogen oxides, carbon monoxide, polycyclic aromatic hydrocarbons, toxic metals, and noise, we considered *t,t*-MA-U as a proxy-biomarker of the overall traffic exposure and not as a mere biomarker of benzene exposure. The concentrations of *t,t*-MA-U ranged from 3 to 2008 µg/L in our group of non-smoking adolescents, which is relatively high in light of studies that investigated the levels of *t,t*-MA-U in populations occupationally exposed to traffic. In Genoa (Italy), the concentrations of *t,t*-MA-U ranged from <10 to 2014 µg/L among non-smoking bus drivers and from <10 to 398 µg/L among non-smoking referents [12]. In Milan, the *t,t*-MA-U range was <10 to 1400 µg/L among non-smoking traffic policemen and <10 to 576 µg/L among referents [12]. The large between-subject differences and the relatively high concentrations of *t,t*-MA-U support the reliability of this metabolite of benzene as a proxy-biomarker of traffic exposure in our study.

Several mechanisms of the neurobehavioral effects of traffic-related air pollution have been investigated. In rodents, it has been shown that ultrafine particles translocate from the nose along the olfactory nerve to the olfactory bulb and other regions of the brain [36,37]. Moreover, particles translocate from the lungs into the blood from which they can reach the brain by crossing the blood-brain barrier [38,39]. 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 [40,41]. The presence of particles in the brain may cause a number of effects including microglial activation [42], oxidative stress [43-45], proinflammatory cytokine response [45], neuronal death [42,44], and changes in neurotransmission [43]. 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 a production of leucocytes and platelets in the bone marrow and an activation of the vascular endothelium [46,47]. Such a systemic response may also affect the brain [48,49]. Studies in rodents exposed to air pollution showed changes in the level and turnover of neurotransmitters [9,50] and gene expression [51,52], an increased level of oxidative stress [8,53], and a pro-inflammatory cytokine response [7,52,54]. Additionally, 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 [40,55].

Our study has several strengths. First, due to the use of *t,t*-MA-U as an internal biomarker of exposure, our traffic exposure assessment covered the exposures at different locations and the commute-related exposure. Second, large between-subject differences in exposure were present, enhancing the ability of the study to accurately assess exposure and to observe exposure effects. Third, we applied multivariate models to estimate the association between *t,t*-MA-U and multiple parameters measuring the same neurobehavioral domain. This approach avoided the problem of multiple testing and could be expected to increase the statistical power.

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. In order to deal with this problem, we excluded smokers from the analysis and accounted for the blood lead concentrations in the analysis. Importantly, the distributions of the education level of the parents, family income, 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 not important confounders of the associations between *t,t*-MA-U and neurobehavioral performance.

CONCLUSIONS

Traffic exposure, as reflected by the concentrations of *t,t*-MA-U, a metabolite of benzene in urine, was associated with a decrease in sustained attention in adolescents. We found no evidence that the association between *t,t*-MA-U and neurobehavioral performance was modified by the education level of the parents or family income.

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Chapter 6

Short- versus long-term air pollution exposure in association with attention, memory, and visual information processing in primary school children

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ABSTRACT

Introduction: Air pollution has been identified as neurotoxic but data on the relevance of different time windows of exposure is scarce. We investigated the association between neurobehavioral performance and short- and long-term air pollution exposure in primary school children.

Methods: Children were examined three times and the average time between two consecutive measurements was 41 days. We studied three neurobehavioral domains: attention (Stroop and Continuous Performance tests), short-term memory (Digit Span Forward and Backward tests), and visual information processing speed (Digit-Symbol and Pattern Comparison tests). We used mixed models to investigate the associations between these neurobehavioral domains and air pollution exposure. The primary indicator of acute exposure was the ambient concentration of ultrafine particulate matter (UFP) in the morning at the examination day. The average past-year and lifetime residential concentrations of NO_2, $PM_{2.5}$, and PM_{10}, distance-weighted traffic density, and distance to the nearest major road were used to assess the long-term exposure.

Results: 334 children participated in the study (mean age: 10.2 years). The total number of examinations was 959. The median UFP concentration at the examination day was 29,800 particles/cm^3 (IQR: 21,600 - 38,100) and the median past-year concentration of $PM_{2.5}$ was 15.7 $\mu g/m^3$ (IQR: 15.2 - 16.3). We found no evidence of an association between acute changes of the UFP level and the neurobehavioral domains studied. An IQR range increase in residential exposure was associated with a decrease in attention of 0.133 SD (95% CI: -0.239 to -0.026) for the past-year $PM_{2.5}$ concentrations, of 0.098 SD (95% CI: -0.185 to -0.010) for the past-year PM_{10} concentrations, and of 0.106 SD (95% CI: -0.200 to -0.012) for the average lifetime PM_{10} concentrations. However, none of the associations between residential long-term air pollution exposure and the neurobehavioral domains studied remained statistically significant after correction for multiple testing and in a sensitivity analysis correcting for the school attended by the children.

Conclusions: Acute changes in the air pollution level were not associated with attention, short-term memory, or visual information processing speed in primary school children. Consistent with previous research, we found some evidence of an association between long-term particulate air pollution exposure and attention.

INTRODUCTION

Air pollution is a complex mixture of gases and solid particles. There is a growing amount of evidence that air pollution exposure is neurotoxic. Experimental studies in rodents demonstrated a wide range of effects of air pollution exposure on the central nervous system including a pro-inflammatory cytokine response, glial activation, oxidative stress, changes in gene expression, and changes in the level and turnover of neurotransmitters [1-6].

Epidemiological studies suggest that the neurotoxic effects of air pollution translate into an observable deterioration of cognitive performance. In children from Boston with a mean age of approximately 10 years, average lifetime residential levels of black carbon were inversely associated with attention, memory, learning, and intelligence [7,8]. In two cohort studies, perinatal air pollution exposure as assessed by personal polycyclic aromatic hydrocarbons monitors was inversely associated with intelligence at early childhood [9-11]. Negative associations between neurobehavioral performance of children and indicators of long-term air pollution exposure have also been reported by cross-sectional studies [12,13]. In adolescents, traffic exposure as indicated by a factor that combined information about traffic density, time spent in traffic, and the concentrations of *trans,trans* muconic-acid in the urine was negatively associated with sustained attention [14].

The neurobehavioral effects of a short-term air pollution exposure (i.e., exposure over the past days) have not been extensively studied to date. The aim of this study was to investigate whether neurobehavioral performance was associated with short- and long-term air pollution exposure in primary school children.

METHODS

Study population

This study was a part of the COGNAC (COGNition and Air pollution in Children) study. Between 2011 and 2013, we invited 770 children (grades three to six) from three primary schools in Flanders (Belgium) to participate in the study. All three schools were located in an area with a substantial amount of traffic (Figure 1). The parents of all participants filled out a questionnaire including information about the current and previous residential addresses, the socioeconomic status of the family, and the smoking behavior of the family members, and provided informed consent for participation.

Out of the 770 children that we invited, 334 (43%) agreed to participate in the study, had the questionnaire filled in and underwent at least one examination. Of the 334 children who participated in our study, 295 (88%) were examined three times, 35 (11%) were examined two times, and 4 (1%) were examined once, amounting to a total number of examinations of 959. The examinations took place between December 2011 and February 2014 on Monday, Tuesday, Thursday, and Friday between 8:30am and 2:10pm. The average (SD) time between two consecutive examinations was 41 (23) days. The three neurobehavioral examinations of a certain child were scheduled for the same time of the day. In some cases, examining a certain child three times at exactly the same time was not possible due to school activities. The average (SD) difference in the time of the day between two examinations of a certain child was 24 (48) min.

The neurobehavioral examination lasted approximately 20 min. The room where the examination took place was quiet and was frequently ventilated in order to prevent accumulation of CO_2. In Flanders, primary school children play at playgrounds outside schools during the breaks between the lessons in all seasons of the year. Consequently, all participants spent some time outdoors before the examination.

Our study protocol was approved by the medical ethical commission of the Hasselt University and the Oost-Limburg Hospital.

Air quality model

The RIO model is a land-use regression model [15]. It estimates hourly concentrations of pollutants in a 3 × 3 km grid using data from the measurement stations and land use data. On average, the measurement stations in Belgium are situated approximately 25 km apart from each other. The IFDM (Immission Frequency Distribution Model) calculates the concentrations of pollutants at a specific location based on information about traffic and industrial emissions in the surroundings and meteorological data such as wind speed, wind direction, and temperature [16,17]. In order to accurately estimate the concentrations of air pollutants at specific locations, the RIO and IFDM models were coupled as described in detail elsewhere [16,17]. In a recent validation study, this modeling approach explained 68% and 84% of the temporal variability of the hourly means for, respectively, NO_2 and PM_{10} [17].

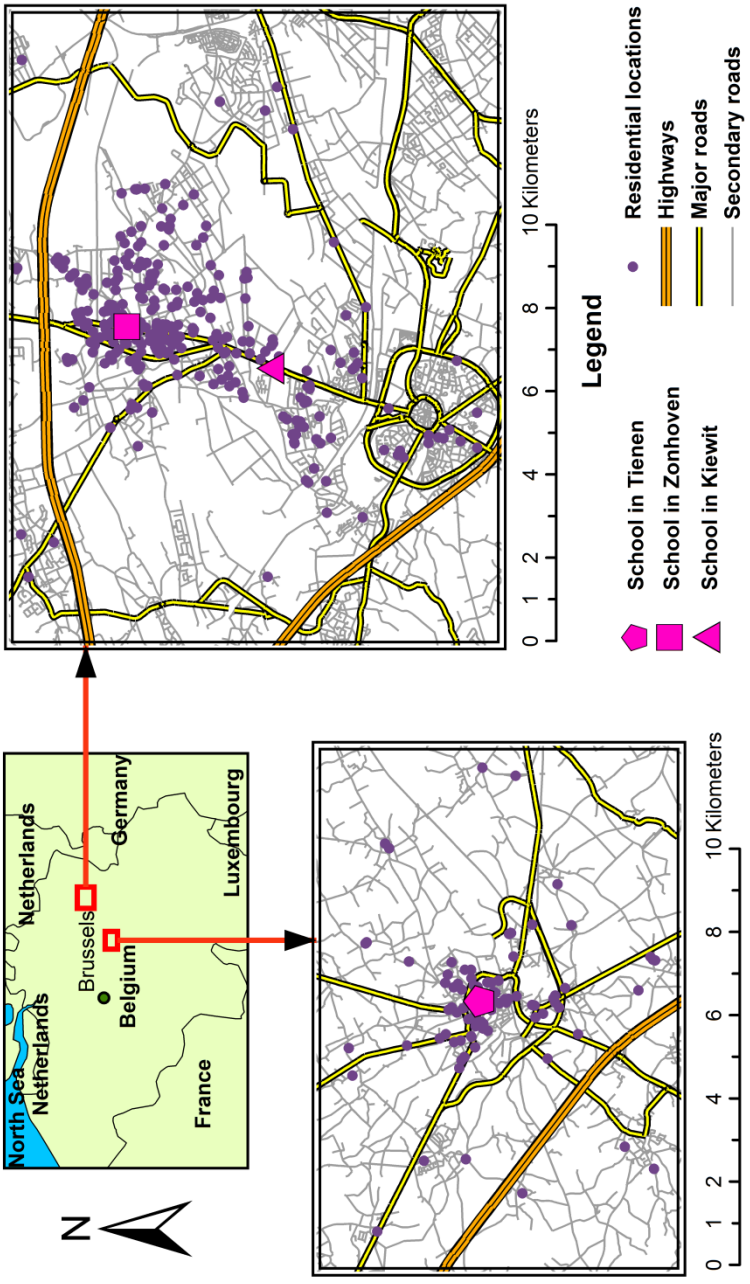

FIGURE 1. Study area.

Traffic indicators

We used Geographical Information System (ArcGIS version 10.0) to calculate distances to the nearest major road and to evaluate the distance-weighted traffic density (DWTD) as described elsewhere [14]. Briefly, the impact of traffic on air quality at a specific location was modeled as a function of the distance to the road according to the following Gaussian model:

$$\frac{1}{0.4*\sqrt{2\pi}} exp\left[-\frac{1}{2} * \frac{\left({distance}/{150}\right)^2}{(0.4)^2}\right].$$

The shape of the formula reflects the assumption that 96% of the emissions disperse at 150 meters from the road, which is based on studies that investigated the dispersion of vehicle exhaust pollutants [18,19]. We calculated the DWTD for each location by adding up the traffic density in the neighborhood weighted by the distance. Information about the average number of vehicles on highways and other major roads in 2010 was available from a network of measuring stations run by the Department of Mobility and Public Works. These traffic counts accurately reflect the traffic intensities across the lifetime of the participants because the structure of highways and other major roads in the study area did not undergo substantial changes between 1999 and 2014. We considered highways and other national roads as major roads.

Short-term exposure indicators

We used portable devices Philips Aerasense NanoTracer and AEROCET 531 to monitor the concentrations of, respectively, ultrafine particles (UFPs) and particulate matter (PM) at the day of the examination. We measured the air pollution level on the playground outside the school in the morning preceding the examination day and during the breaks between the lessons. Additionally, we measured the concentrations of the pollutants in the room where the examinations took place.

As a primary exposure indicator, we used the 10-minutes average concentrations of UFPs outside the school in the morning preceding the school day. We considered this indicator as the most important one because small particles might have the greatest neurotoxic potential and because the concentrations in the morning were strongly predictive of the concentrations throughout the whole study day inside and outside the school. Because children spent most of the time during the school day inside the school, we additionally considered the average UFP concentrations between 9am and 12am in the classroom where the examination took place.

Additionally, we calculated the exposure indicators for PM_{10} and $PM_{2.5}$ the same way as for UFP. Because we measured the concentrations of pollutants only at the day of the examination we also used RIO-IFDM predictions as secondary exposure indicators. We considered the daily mean concentrations of

NO_2, $PM_{2.5}$, and PM_{10} two days before the examination, one day before the examination, and at the day of the examination. An overview of the exposure indicators used in the study is presented in Table 1.

TABLE 1. Overview of exposure indicators.

	UFP	$PM_{2.5}$	PM_{10}	NO_2	DWTD	Road[a]
Short-term (at school)						
Outdoor measurements at the examination day	x	x	x			
Indoor measurements at the examination day	x	x	x			
RIO-IFDM estimates of the daily means, lag0		x	x	x		
RIO-IFDM estimates of the daily means, lag1		x	x	x		
RIO-IFDM estimates of the daily means, lag2		x	x	x		
Long-term (residential)						
RIO-IFDM estimates of the average past-year concentrations		x	x	x	x	x
RIO-IFDM estimates of the average lifetime concentrations		x	x	x	x	x

[a] Distance to the nearest major road.

Long-term exposure indicators

As indicators of the long-term air pollution exposure, we used RIO-IFDM estimates of the residential concentrations of NO_2, $PM_{2.5}$, and PM_{10}, residential DWTD, and residential distance to the nearest major road. For each indicator, we estimated the past-year and lifetime exposure levels. In order to achieve this, we used information about the current and previous residential addresses, the years of moving, and the proportion of time spent at each location in the case of children living at two locations at a certain time point (e.g., because of divorce of the parents). In the case of the past-year exposure, we used the average concentrations of NO_2, $PM_{2.5}$, and PM_{10} during the period of 365 days preceding the first neurobehavioral examination. When a child had more than one residential address at the moment of the study, we calculated a weighted average using the proportion of time spent at each location as weights. In order to estimate the lifetime residential exposure, we calculated weighted averages using the proportion of time a child lived at a certain location as weight. For NO_2, $PM_{2.5}$, and PM_{10}, we used the annual means in these calculations, which were available during the period 2009-2013. For earlier years, we used the means for 2009. This was a good approximation because there was little variability in the annual means of air pollutants for a specific location in our study area. For example, the correlation between the estimates of the mean annual concentrations at residential locations in 2009 and 2013 equaled r=0.98 for NO_2, r=0.95 for $PM_{2.5}$, and r=0.87 for PM_{10}.

Neurobehavioral tests

We administered a computer version of the Stroop Test [20] and the following four tests from the Neurobehavioral Evaluation System 3 (NES3)

battery: Continuous Performance, Digit Symbol, Digit Span, and Pattern Comparison (Figure 2) [21,22].

In the Stroop Test, four buttons are displayed on the screen (yellow, red, blue, and green). During the test, the name of one of these colors appears on the screen, printed in a different color than the name. The task is to touch the button that has the same color as the name, ignoring the print color, as fast as possible. Before the test, eight practice trials take place. Then, 48 test trials follow. The mean reaction time is the average time that passed between the appearance of the name and touching the correct button. This indicator was only calculated when the total number of trails with wrong responses was smaller than or equal to 16.

In the Continuous Performance Test, pictures of animals are displayed on the screen, one at a time, and each for approximately 200msec. The task is to immediately respond to the picture of a cat, and not pictures of other animals, by pressing the spacebar. A new picture is displayed each 1000msec.

The Digit Span Test consists of two parts. In the first part, the task is to reproduce a series of digits after an oral presentation in the order of the presentation. The test starts with a sequence of three digits. In the case of a correct answer, a one digit longer sequence is presented. The test continues until two incorrect answers in a row are given. In the second part of the test, the task is to reproduce digits in the reverse order.

In the Digit-Symbol Test, a row of 9 symbols paired with 9 digits is shown at the top of the screen. The same 9 symbols but in a different order are displayed at the bottom of the screen. During the test 27 digits appear on the screen. When a digit is shown, the task is to, as fast as possible, indicate the symbol, which is paired with this digit, from the row of symbols at the bottom of the screen. A new digit appears only after the correct symbol has been indicated.

In the Pattern Comparison Test, three matrices consisting of 10 x 10 blocks are shown. Two of them are identical. The task is to indicate which pattern is different from the two other patterns. The test includes 25 items.

As indicators of attention, we used the mean reaction time in the Continuous Performance Test and the mean reaction time in the Stroop Test. As indicators of short-term memory, we used the maximum span forward and backward in the Digit Span Test. As indicators of visual information processing speed, we used the total latency in the Digit-Symbol Test and the average latency in the Pattern Comparison Test.

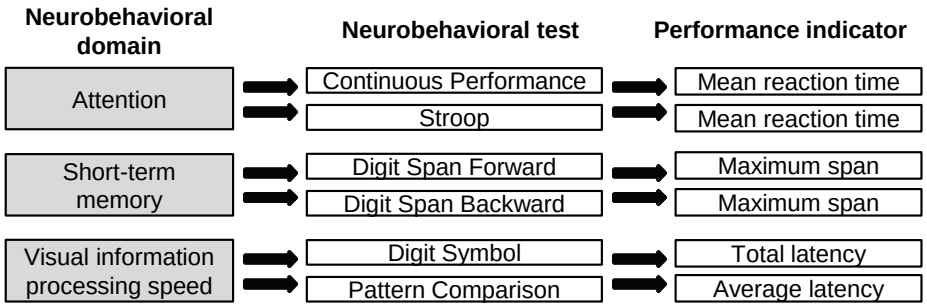

FIGURE 2. Overview of the neurobehavioral indicators.

Statistical analysis

We standardized (i.e., calculated the z-scores) all indicators of the neurobehavioral domains in order to express them on the same scale. The first two categories of education level were merged due to a small number of participants in the first category. We transformed DWTD and distance to the nearest major road logarithmically to reduce the skewness of their distributions.

We applied multivariate mixed models [23] to study the association between the indicators of the short- and long-term air pollution exposure and attention, short-term memory, and visual information processing speed. Estimation of the effects of the short- and long-term exposures required a different strategy to deal with potential confounders. In the case of the short-term exposure, we modeled for each neurobehavioral domain and each exposure indicator the result of person *i* at measurement occasion *j* (*j*=1,2,3) in test *k* (*k*=1,2) as:

$$Y_{i,j,k} = \alpha_k + \beta_W\left(Pollution_{i,j} - \overline{Pollution_i}\right) + \sum_{s=1}^{S}\beta_s^{(1)}X_{s,i}^{(1)} + \sum_{t=1}^{T}\beta_t^{(2)}X_{t,i,j}^{(2)} + b_{i,k} + \varepsilon_{i,j,k},$$

where α_k is the intercept for test *k*, $Pollution_{i,j}$ is the air pollution level for person *i* at measurement occasion *j*, $\overline{Pollution_i}$ is the average air pollution level across the measurements for person *i*, $X_{s,i}^{(1)}$ are person-related covariates for person *i*, $X_{t,i,j}^{(2)}$ denote time-related covariates for person *i* at measurement occasion *j*, $\beta_W, \beta_s^{(1)}$, and $\beta_t^{(2)}$ are the corresponding regression coefficients, $b_{i,k}$ is the random intercept for subject *i* in test *k*, and $\varepsilon_{i,j,k}$ is the measurement error. The subject-specific random intercepts $b_{i,1}$ and $b_{i,2}$ were allowed to be correlated. The measurement errors $\varepsilon_{i,j,k}$ were assumed to be independent and were allowed to have a different variance for different tests and measurement occasions. We explicitly modeled the within-subject changes of air pollution exposure. The regression coefficient β_W represents the effect of acute changes of air pollution on cognitive performance of an individual. The advantage of this model specification is that it excludes the risk of confounding of subject-specific

characteristics and the risk of bias due to the fact that we examined different children in different seasons [24,25]. In order to account for potential confounding of time-varying covariates, our models included day of the week, apparent temperature, and measurement occasion. In order to account for the possibly non-linear effects of apparent temperature, we used restricted cubic splines with 3 knots located at the 5^{th}, 50^{th}, and 95^{th} percentiles. Besides potential confounders, our models included relevant subject-specific covariates in order to reduce the variability of the random intercepts. We used a stepwise selection procedure based on a model including the fixed intercept, the random intercept, and the measurement error with p=0.05 for entry and staying in the model and the following candidate covariates: age, age^2, gender, the level of education of the mother and of the father, the highest category of occupation of either parents, home ownership, and school. As a result of this procedure, age, age^2, and occupation of the parents were included in the model of attention; age and occupation of the parents in the model of short-term memory; and age, age^2, gender, and occupation of the parents in the model of visual information processing.

In the case of the long-term exposure, we modeled for each neurobehavioral domain and each exposure indicator the result of person *i* at measurement occasion *j* in test *k* as:

$$Y_{i,j,k} = \alpha_k + \beta Pollution_i + \sum_{s=1}^{S} \beta_s^{(1)} X_{s,i}^{(1)} + \sum_{t=1}^{T} \beta_t^{(2)} X_{t,i,j}^{(2)} + b_{i,k} + \varepsilon_{i,j,k},$$

where $Pollution_i$ is the air pollution level for person *i* and β is the corresponding regression coefficient. Subject-specific characteristics were potential confounders of the association between long-term air pollution exposure and the neurobehavioral outcomes. Therefore, we included gender, age, age^2, education of the mother, occupation of the parents, home ownership, and passive smoking in the models. Additionally, the model included a time-varying covariate indicating the measurement occasion, which was an important predictor of neurobehavioral performance due to the learning effect.

We also investigated whether the associations between air pollution exposure and the neurobehavioral outcomes were modified by the level of education of the mother by adding the interaction term to the model. We corrected for multiple testing in the analyses involving multiple exposure indicators using a resampling-based approach, which accounts for the fact that individual hypotheses are not independent [26,27]. First, we used parametric bootstrap to create samples $\boldsymbol{Y}^*$ by simulating from the distribution fitted under the null hypothesis (of no association between exposure indicators and a neurobehavioral domain). Next, we calculated for each sample the t-test statistics for each exposure indicator, thereby estimating the joint distribution of the test statistics under the null hypothesis. Finally, we applied a step-down Min P method to calculate p-values adjusted for multiple testing [26]. This procedure controls the familywise error rate (i.e., the probability that one or more null

hypothesis is incorrectly rejected) [26,27]. The reported tests of the statistical significance refer to the individual tests, unless it is indicated that they are corrected for multiple testing. The analysis was performed in SAS (version 9.4).

TABLE 2. Characteristics of the participants.

	N=334
School, N=334[a]	
Kiewit	76 (22.8%)
Tienen	70 (21%)
Zonhoven	188 (56.3%)
Demographic characteristics, N=334[a]	
Boys	165 (49.4%)
Age	10.2 (1.3)
Level of education of the mother, N=327[a]	
No high school diploma	5 (1.5%)
High school diploma	124 (37.9%)
College or university diploma	198 (60.6%)
Level of education of the father, N=323[a]	
No high school diploma	9 (2.8%)
High school diploma	141 (43.7%)
College or university diploma	173 (53.6%)
Highest category of occupation of either parents, N=320[a]	
Unemployed or not qualified worker	27 (8.4%)
Qualified worker, white-collar assistant, or teaching staff	122 (38.1%)
Self-employed, specialist, or member of management	171 (53.4%)
Home ownership, N=330[a]	294 (89.1%)
Passive smoking, N=327[a]	47 (14.4%)

Arithmetic mean (standard deviation) is given for the continuous variables. Count (percent) is given for the categorical variables.
[a] Number of participants for whom information was available.

RESULTS

Study population

Characteristics of the study group are summarized in Table 2. The number of boys and girls was approximately equal. The mean (SD) age was 10.2 (1.3) years. A majority (61%) of the mothers of the children had a college or university diploma (Table 2). The median concentrations of air pollutants in the morning outside the school equaled 29,800 particles/cm^3 for UFP, 13.2 µg/m^3 for $PM_{2.5}$, and 44.3 µg/m^3 for PM_{10} (Table 3). The median residential lifetime exposure equaled 22.2 µg/m^3 for NO_2, 17.0 µg/m^3 for $PM_{2.5}$, and 21.4 µg/m^3 for PM_{10} (Table 4).

TABLE 3. Short-term exposure level during the study period.

	P5	P25	Median	P75	P95
Outdoors					
UFP, 1000 x particles/cm^3	10.0	21.6	29.8	38.1	98.5
$PM_{2.5}$, $\mu g/m^3$	1.7	7.5	13.2	27.0	92.5
PM_{10}, $\mu g/m^3$	7.5	17.7	44.3	75.4	175.5
Indoors					
UFP, 1000 x particles/cm^3	4.3	6.8	9.8	15.2	22.5
$PM_{2.5}$, $\mu g/m^3$	1.6	2.9	5.1	11.6	27.9
PM_{10}, $\mu g/m^3$	16.4	21.6	34.6	76.1	164.9

TABLE 4. Long-term exposure level.

	P5	P25	Median	P75	P95
Past-year					
NO_2, $\mu g/m^3$	17.2	19.5	21.2	22.6	25.0
$PM_{2.5}$, $\mu g/m^3$	14.3	15.2	15.7	16.3	17.0
PM_{10}, $\mu g/m^3$	19.6	20.6	21.2	22.2	24.0
DWTD, km/day	58	85	124	436	2125
Distance to major road, m	30	133	340	832	1692
Lifetime					
NO_2, $\mu g/m^3$	18.2	20.7	22.2	23.7	25.9
$PM_{2.5}$, $\mu g/m^3$	15.8	16.5	17.0	17.3	17.7
PM_{10}, $\mu g/m^3$	19.7	20.8	21.4	22.3	24.0
DWTD, km/day	60	87	125	553	2016
Distance to major road, m	32	145	409	843	1632

DWTD: distance-weighted traffic density.

Associations between short-term exposure and neurobehavioral domains

The associations between short-term air pollution exposure and the neurobehavioral domains studied were not statistically significant. An IQR increase in the UFP levels outside the school was associated with a 0.006 standard deviations (SD) decrease in attention (95% Confidence Interval: -0.028 to 0.016, p=0.57), 0.021 SD decrease in short-term memory (95% CI: -0.050 to 0.008, p=0.15), and 0.001 SD decrease in visual information processing speed (95% CI: -0.019 to 0.018, p=0.93, Table 5).

In the secondary analysis, an IQR increase of the PM_{10} concentrations outside the school was associated with a 0.033 SD decrease in visual information processing speed (95% CI: -0.065 to -0.001, Table 5). An IQR range increase of the $PM_{2.5}$ concentrations inside the school was associated with a 0.102 SD decrease in short-term memory (95% CI: -0.192 to -0.013). However, accounting for the fact that 14 secondary exposure indicators were considered, no evidence of association between short-term air pollution exposure and the neurobehavioral domains studied was apparent (p>0.99 for attention, p>0.20 for short-term memory, and p>0.32 for visual information processing speed).

We found no evidence that the associations between short-term air pollution exposure and the neurobehavioral domains were modified by the level of education of the mother (p>0.39).

TABLE 5. Associations between short-term air-pollution exposure and neurobehavioral domains.

Exposure indicator	Attention		Short-term memory		Visual information processing	
	Effect size	95% CI	Effect size	95% CI	Effect size	95% CI
Main analysis						
UFP-outdoors	-0.006	-0.028 to 0.016	-0.021	-0.050 to 0.008	-0.001	-0.019 to 0.018
Secondary analysis						
$PM_{2.5}$-outdoors	0.007	-0.022 to 0.035	0.010	-0.028 to 0.047	-0.010	-0.034 to 0.014
PM_{10}-outdoors	-0.004	-0.040 to 0.031	0.008	-0.041 to 0.056	-0.033	-0.065 to -0.001
UFP-indoors	-0.013	-0.065 to 0.039	0.002	-0.067 to 0.071	-0.006	-0.050 to 0.039
$PM_{2.5}$-indoors	0.015	-0.054 to 0.083	-0.102	-0.192 to -0.013	-0.038	-0.098 to 0.022
PM_{10} -indoors	0.014	-0.065 to 0.093	-0.085	-0.185 to 0.015	-0.029	-0.096 to 0.037
NO_2-modeled-lag0	-0.006	-0.052 to 0.040	-0.011	-0.071 to 0.048	-0.014	-0.054 to 0.026
$PM_{2.5}$-modeled-lag0	0.008	-0.039 to 0.056	-0.018	-0.080 to 0.044	-0.013	-0.054 to 0.027
PM_{10}-modeled-lag0	0.004	-0.040 to 0.048	-0.014	-0.072 to 0.043	-0.015	-0.052 to 0.023
NO_2-modeled-lag1	-0.001	-0.045 to 0.044	-0.010	-0.068 to 0.048	0.029	-0.008 to 0.067
$PM_{2.5}$-modeled-lag1	-0.009	-0.051 to 0.034	-0.025	-0.081 to 0.030	-0.008	-0.043 to 0.028
PM_{10}-modeled-lag1	-0.011	-0.049 to 0.026	-0.023	-0.072 to 0.026	-0.004	-0.035 to 0.027
NO_2-modeled-lag2	-0.022	-0.075 to 0.032	0.011	-0.058 to 0.080	0.000	-0.045 to 0.046
$PM_{2.5}$-modeled-lag2	-0.007	-0.052 to 0.039	0.009	-0.051 to 0.068	-0.018	-0.057 to 0.021
PM_{10}-modeled-lag2	-0.006	-0.052 to 0.040	0.001	-0.058 to 0.061	-0.014	-0.052 to 0.025

The table shows the change in the neurobehavioral domains associated with an IQR range increase in exposure. The results are expressed in the number of standard deviations. All models included the following potential confounders: day of the week, apparent temperature, and measurement occasion. Additionally, the subject-specific covariates age, age^2, and occupation of the parents were included in the model of attention; age and occupation of the parents in the model of short-term memory; and age, age^2, gender, and occupation of the parents in the model of visual information processing.

Associations between long-term exposure and neurobehavioral domains

An IQR range increase in residential exposure was associated with a decrease in attention of 0.133 SD (95% CI: -0.239 to -0.026) for the past-year $PM_{2.5}$ concentrations, of 0.098 SD (95% CI: -0.185 to -0.010) for the past-year PM_{10} concentrations, and of 0.106 SD (95% CI: -0.200 to -0.012) for the average lifetime PM_{10} concentrations, correcting for gender, age, age^2, education of the mother, occupation of the parents, home ownership, and passive smoking (Figure 3). The p-values adjusted for multiple testing equaled, respectively, 0.10, 0.15, and 0.15. We found no evidence that the associations between air pollution exposure and the neurobehavioral domains studied were modified by the level of education of the mother ($p>0.07$).

Sensitivity analysis

Because the attended school may be associated with cognitive capabilities of pupils, we performed a sensitivity analysis that included school as a covariate. Although school was not a significant predictor of attention ($p=0.14$ in a model adjusting for gender, age, age^2, education of the mother, occupation of the parents, and home ownership), adding it to the models changed the strength of the association between long-term residential air pollution exposure

and attention. After the additional correction for school the estimates equaled -0.097 SD (95% CI: -0.220 to 0.026) for the past-year $PM_{2.5}$ concentrations, -0.052 SD (95% CI: -0.189 to 0.086) for the past-year PM_{10} concentrations, and -0.086 SD (95% CI: -0.227 to 0.055) for the average lifetime PM_{10} concentrations.

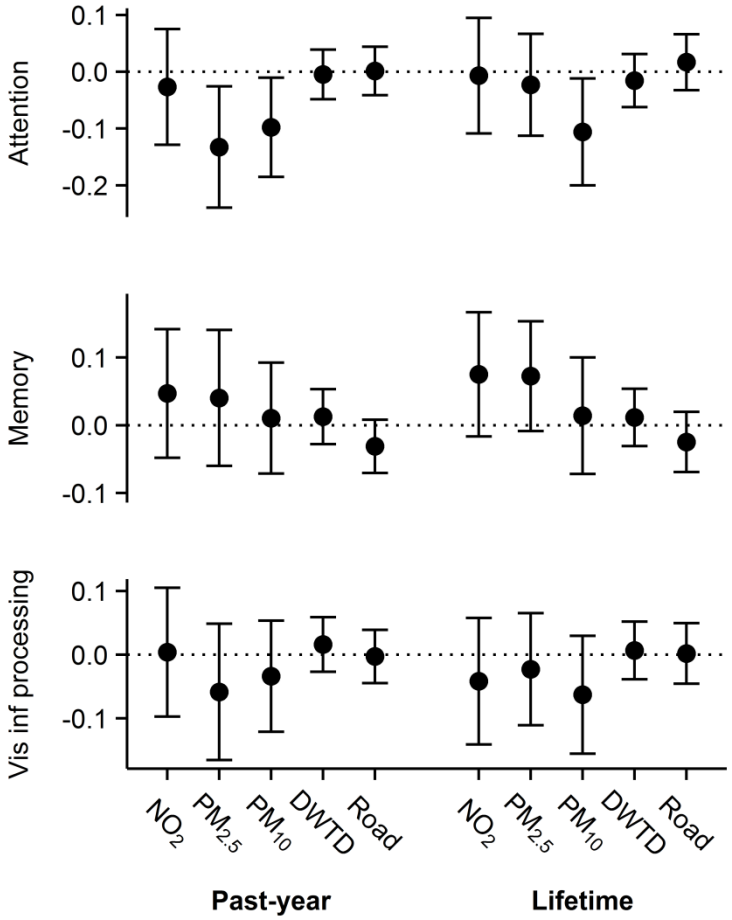

FIGURE 3. Associations between long-term exposure and neurobehavioral domains.

The figure shows the change in the neurobehavioral domains associated with an IQR range increase in NO_2, $PM_{2.5}$, and PM_{10}, a two-fold increase in DWTD, and a two-fold decrease in the distance to the nearest major road. The effects are expressed in the number of standard deviations. All models included gender, age, age^2, education of the mother, occupation of the parents, home ownership, passive smoking, and measurement occasion.

DISCUSSION

In the first panel study investigating the association between air pollution exposure and neurobehavioral performance to date, we found no evidence that acute changes in the level of air pollution were associated with attention, short-term memory, or visual information processing speed in primary school children. We found some evidence of associations between long-term residential particulate matter exposure and attention. However, the observed associations did not remain statistically significant in a sensitivity analysis correcting for school attended by the children, which was a potential confounder. Moreover, the associations were not statistically significant after correction for multiple testing.

Several recent studies reported an inverse association between air pollution exposure and attention-related outcomes. Recently, we observed a negative association between sustained attention measured using the Continuous Performance Test and traffic exposure as assessed by the concentrations of traffic density, time spent in traffic, and the concentrations of *trans, trans* muconic-acid [14]. In a Boston study of 174 children between 7 and 14 years of age, residential concentrations of black carbon were negatively associated with attention measured by the Continuous Performance Test [8]. A Chinese study reported that children whose school was located in an area with a low traffic density performed better in the Continuous Performance Test than those from a school with much traffic-related air pollution [13]. In elderly, the residential distance to a busy road was associated with attention, as assessed by the Stroop Test [28]. In an Indian study, the prevalence of ADHD was higher in urban children than in controls living in an area with little air pollution [29]. Another study indicated that the average residential elemental carbon concentrations during the first year of life were associated with a higher risk of hyperactivity at 7 years of age, as assessed by the Behavioral Assessment System for Children, Parent Rating Scale [30]. In line with these results, our study found an indication that a long-term residential particulate matter exposure may be associated with attention in primary school children.

When particulate matter enters the lungs, they release inflammatory mediators to the systemic circulation [31,32]. Additionally, small particles translocate into the systemic circulation, which may also lead to an increased level of systemic inflammation [33]. As demonstrated by a large body of evidence, systemic inflammation adversely affects the brain [34,35]. Besides their effect on the level of systemic inflammation, nanoparticles can also cause harm in a more direct way by translocating to the brain through the blood-brain barrier or through the olfactory nerve [36,37]. When adverse changes occur in the brain, brain activity associated with a given function can move to a different location. It is possible that a low-level air pollution exposure exerts cognitive

effects only with a prolonged exposure due to the plasticity of the brain. The results of our study may reflect that a long-term air pollution exposure is more relevant for neurobehavioral functioning than acute changes in the air pollution level.

The repeated measures study design that we used is an efficient approach to accurately estimate the short-term effects of air pollution. The half-width of the 95% confidence intervals of the change in neurobehavioral domains associated with an IQR increase of the UFP concentrations equaled 0.022 SD for attention, 0.029 SD for short-term memory, and 0.019 SD for visual information processing speed. This high precision implies that our study had a large power to detect relatively small changes in neurobehavioral performance associated with acute changes of the air pollution level. As expected from our study design, the precision was lower for the long-term exposure effects. For example, the half-width of the 95% confidence intervals of the change in neurobehavioral domains associated with an IQR increase in the past-year residential $PM_{2.5}$ exposure equaled 0.102 SD for attention, 0.095 SD for short-term memory, and 0.101 SD for visual information processing speed.

Our study has several strengths. First, the risk of bias in the analysis of the short-term effects of air pollution was small owing to the panel study design and the statistical approach used. More specifically, we excluded the risk of reverse causality and confounding of person-related characteristics. Second, we were able to estimate the change in neurobehavioral performance associated with the short-term air pollution exposure with a high precision. Equivalently, out study had a large power to detect relatively small neurobehavioral changes. Third, we measured the outdoor and indoor concentrations of air pollutants at the locations of the schools at the examination days. Evaluating the exposure this way is more accurate than by using air quality models.

The major weakness of the analysis of the long-term air pollution exposure was the small variability in the level of exposure. For example, the IQR of the average lifetime PM_{10} exposure equaled 1.5 $\mu g/m^3$. This small variability had a negative impact on the power of this analysis.

CONCLUSIONS

This is the first panel study of the neurobehavioral effects of air pollution exposure. Despite the large power to detect relatively small effects, our study provided no evidence of an association between acute changes of the level of air pollution and neurobehavioral performance. Consistent with previous studies, we found some evidence of a negative association between residential particulate matter exposure and attention. The results of our study may reflect that a long-

term air pollution exposure is more relevant for neurobehavioral performance than acute changes in the air pollution level.

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Chapter 7

General discussion*

*Partly based on: Kicinski M, Nawrot T. Neurobehavioral effects of air pollution in children. In: *Environmental factors in neurodevelopmental and neurodegenerative disorders*. Edited by: Costa L, Aschner M; Elsevier; 2015.

BROMINATED FLAME RETARDANTS

Discussion of study findings

A two-fold increase of the sum of serum lower-brominated PBDE congeners 47, 99, 100, and 153 was associated with a decrease of the number of taps with the preferred-hand in the Finger Tapping Test by 5.31 (95% CI: 0.56 to 10.05, p=0.029). We found no evidence of an association between PBDEs and outcomes measuring sustained attention, short-term memory, and visual information processing speed. We also did not observe any consistent associations between neurobehavioral performance and HBCD or TBBPA.

The association between exposure to PBDEs and manual motor speed is in line with animal studies that observed effects of PBDEs on motor activity of animals [1-4]. Recently, several epidemiological studies investigated the motor effects of PBDEs. A study of 234 children from California reported a negative association between the concentrations of PBDEs in maternal prenatal serum and the number of taps with the preferred-hand in the finger tapping test at age 5 [5]. However, the association was no longer statistically significant at age 7. The study also observed a negative association between perinatal exposure to PBDEs and performance in the Pegboard Test, which involves placing pins in holes as quickly as possible, at age 5 and 7 [5]. However, perinatal exposure to PBDEs was not associated with psychomotor development at early childhood as indicated by the Psychomotor Development Index from the Bayley Scales of Infant Development in three small prospective cohort studies [6-8]. A study in 144 elderly found no evidence of an association between the sum of serum concentrations of PBDEs and the motor function evaluated using the Finger Tapping Test, the Pegboard Test, and the Static Motor Steadiness Test [9]. A possible explanation why a negative association between exposure to PBDEs and neurobehavioral performance is observed only in some epidemiological studies is their small sample size and the resulting low power.

Public health implications

In the beginning of this century, several measures have been taken to limit the use of PBDEs, including:

- the European Union ban of products containing more than 0.1% penta-BDE and octa-BDE, two common commercial PBDE mixtures of lower-brominated PBDEs, in 2004 [10],
- the voluntary phase-out of penta-BDE and octa-BDE by the industry in the US and Canada in 2004 and 2005 [10],
- the phase-out of penta-BDE by Chinese producers (octa-BDE was never manufactured in China) [11] in 2004, and

- the listing of penta-BDE and octa-BDE as persistent organic pollutants by the Stockholm Convention for Persistent Organic Pollutants in 2009 [12].

Studies on the neurobehavioral effects of PBDEs including our study add to the body of evidence [13,14], which indicates that these policies were needed to protect human health.

HBCD and TBBPA are still in use today. In this PhD project, we found no evidence of an association between exposure to these chemicals and neurobehavioral performance.

TOXIC METALS

Discussion of study findings

Lead

In a study of 606 adolescents, we found no evidence of an association between the blood lead level and sustained attention, short-term memory, or manual motor speed. The mean blood lead level in our study equaled 13.8 µg/L and 95% of the study participants had blood lead concentrations lower than 28.1 µg/L.

Lead impairs intelligence and specific cognitive functions of children at exposure levels that were present before the phase-out of lead from gasoline and paints [15-17]. There is also a substantial amount of evidence showing that lead is associated with impaired cognition at blood lead levels below 100 µg/L [18-20]. Additionally, two recent studies observed a negative association between neurobehavioral performance and blood lead at levels of exposure comparable to those in our study [21,22]. The exposure level at which no neurotoxic effects of lead are observable remains to be established by future studies.

Copper

Blood copper was associated with sustained attention and short-term memory in a population of adolescents with normal copper levels. A two-fold increase in blood Cu was associated with a 0.37 standard deviations decrease in sustained attention (95% CI: -0.67 to -0.07) and a 0.39 standard deviations decrease in short-term memory (95% CI: -0.70 to -0.07). The stratified analysis for gender revealed significant associations between copper and neurobehavioral performance only for girls. A major limitation of our study is that we measured the total blood copper level. This level only partly reflects the amount of free copper, which may have the greatest neurotoxic potential.

Copper causes damage to the central nervous system when a large amount of this metal accumulates in tissues, as evidenced in patients with Wilson disease [23]. Preliminary results suggest that copper may be inversely associated with cognitive performance for normal copper levels [24-26]. Our observation of a negative association the between blood copper level and sustained attention and short-term memory in adolescents adds to this preliminary evidence.

Other toxic metals

We observed no associations between methylmercury, arsenic, manganese, cadmium, nickel, or thallium and sustained attention, short-term memory, or manual motor speed in adolescents. In other words, in this PhD project we found no evidence of neurotoxic effects of these metals for a low-level exposure in adolescents.

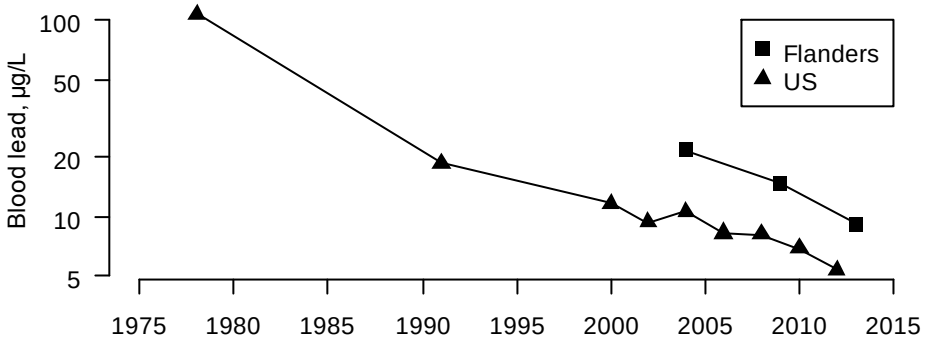

FIGURE 1. Evolution of blood lead level in adolescents.

The figure shows the geometric means of the blood lead level in the populations of Flemish and American adolescents. For Flanders, the data come from studies of Steunpunt Milieu en Gezondheid in grade nine high school students (approximately 15 years of age) [27,28]. For the US, the mean concentrations in adolescents between 14 and 16 years of age examined as a part of the National Health and Nutrition Examination Survey are given [29].

Public health implications

Lead

The case of lead demonstrates that environmental regulations can efficiently reduce human exposure to chemicals. In the middle of the 20th century, the cases of lead poisoning were common [16,17]. Due to restrictions in the use of lead, the exposure level in the developed countries substantially decreased in the past 40 years (Figure 1). This PhD project contributed to the research investigating whether lead is neurotoxic at the current exposure levels. We found no evidence of an association between lead exposure and neurobehavioral performance in Flemish adolescents with the mean blood lead

level 13.8 µg/L. However, other recent studies observed a negative association between blood lead and cognitive outcomes in children at comparable levels of exposure [21,22], suggesting that lead exposure still represents a public health risk in countries with stringent lead regulations.

Copper

Human studies investigating the effects of copper at low exposure levels are scarce. Because copper is an essential element, the public health relevance of our observation of a negative association between the blood copper level and sustained attention and short-term memory in adolescent girls remains to be elucidated by future research.

AIR POLLUTION

Discussion of study findings

As a part of this PhD, we performed several studies investigating the association between traffic-related air pollution exposure and neurobehavioral performance. In a cross-sectional study of 606 adolescents examined between 2008 and 2011 (Chapter 4), we found evidence of an association between traffic exposure and sustained attention. In this study, exposure was evaluated with a factor based on a biomarker of benzene (*t,t*-MA-U) and the amount of contact with traffic (using distance-weighted traffic density and time spent in traffic). A one SD increase in this factor was associated with a 0.26 SD decrease in sustained attention (95% BCI: -0.02 to -0.51).

Between 2012 and 2015, another cross-sectional study of adolescents was performed. We pooled the data from this study with the data gathered between 2008 and 2011 to investigate whether traffic exposure was associated with sustained attention and short-term memory in the analysis based on an extended study group including 931 non-smoking adolescents (Chapter 5). In this analysis, a ten-fold increase in *t,t*-MA-U was associated with a 0.17 SD decrease in sustained attention (95% CI: -0.31 to -0.04), supporting the existence of an inverse association between traffic exposure and neurobehavioral performance.

In primary school children, we used a panel study design (Chapter 6), meaning that each study participant was examined multiple times during a short period of time. 334 children participated in the study and the total number of measurements was 959. By using a panel study design, we could investigate the short-term effects of air pollution exposure without the risk of reverse causality and confounding of person-related characteristics. The study provided no

evidence of associations between short-term air pollution exposure and attention, short-term memory, or visual information processing speed in primary school children. However, we found an indication of an inverse association between the residential long-term air pollution levels and neurobehavioral performance. An IQR range increase in exposure was associated with a decrease in attention of 0.133 SD (95% CI: -0.239 to -0.026) for the average past-year $PM_{2.5}$ concentrations, of 0.098 SD (95% CI: -0.185 to -0.010) for the average past-year PM_{10} concentrations, and of 0.106 SD (95% CI: -0.200 to -0.012) for the average lifetime PM_{10} concentrations. However, these associations did not remain statistically significant in a sensitivity analysis correcting for school attended by the children, which was a potential confounder. Additionally, the associations were not statistically significant after correction for multiple testing related to the use of different indicators of the long-term air pollution exposure (Chapter 6).

At this moment, more than twenty epidemiological studies exist, which have investigated the association between air pollution exposure and neurobehavioral outcomes in children (Table 1 and Table 2). A large part of these studies considered the average level of exposure during childhood or the average exposure at the year of the examination (Table 1). Other studies focused on the effects of a perinatal exposure on neurodevelopment (Table 2). Most studies used residential concentrations of pollutants as exposure indicators. A majority of studies reported evidence of a negative association between air pollution exposure and neurobehavioral outcomes. Additionally, several experimental animal studies that considered environmentally relevant exposure levels provided support to the hypothesis of detrimental effects of air pollution on the neurobehavioral function [30-32]. Our observation of a negative association between traffic exposure and sustained attention in adolescents adds to the evidence of neurobehavioral effects of traffic-related air pollution.

To our best knowledge, our study in primary school children was the first to explicitly investigate the neurobehavioral effects of day-to-day changes in the level of air pollution. Despite a good precision of our estimates, which implies a high power, we found no evidence of such effects. However, consistent with previous studies, we found some indication of a negative association between long-term residential particulate matter exposure and attention. The results of our study in primary school children may reflect that a long-term air pollution exposure is more relevant for neurobehavioral performance than day-to-day changes in the air pollution level.

Table 1. Air pollution exposure in childhood and neurobehavioral performance.

Reference	Study group	Age	Study design	Results
Calderón-Garcidueñas et al., 2008 [33]	Children from Mexico-City (N=55) and controls (N=18) matched by socioeconomic status	Mean: 10.7 years	Cross-sectional	- Children from Mexico-City but not controls showed cognitive deficits as indicated by the total score for 7 of the 12 subscales (Information, Similarities, Vocabulary, Comprehension, Digit Span, Object assembly, and Coding) of the Wechsler Intelligence Scale for Children–Revised.
Calderón-Garcidueñas et al., 2011 [34]	Children from Mexico-City (N=20) and controls (N=10) matched by age and socioeconomic status	Mean age at baseline: 7 years	Prospective cohort	- Weaker performance of children from Mexico City in 5 of the 12 subscales of the Wechsler Intelligence Scale for Children-Revised (Arithmetic, Vocabulary, Digit Span, Picture Completion, and Coding) at baseline and/or after a 1 year follow-up. - The comparisons of the evolution of cognitive performance over time not reported.
Chiu et al., 2013 [35]	A subsample from the Boston-based birth cohort (N=174)	7-14 years	Retrospective cohort	- Lifetime residential black carbon levels negatively associated with sustained attention as indicated by the mean reaction time and the number of commission errors in the Continuous Performance Test, adjusting for age, gender, child IQ, blood lead level, maternal education, pre-and postnatal tobacco smoke exposure, and community-level social stress.
Clark et al., 2012 [36]	Children from the RANCH project attending schools around London's Heathrow airport (N=719)	9-10 years	Cross-sectional	- Mean annual NO_2 concentrations at schools not associated with reading comprehension, recognition memory, information recall, conceptual recall, or working memory.
Freire et al., 2010 [37]	A subsample from a birth cohort from the south of Spain (N=210)	4-5 years	Cross-sectional	- Residential mean annual NO_2 concentrations not associated with any of the 11 cognitive domains from the McCarthy Scales of Children's Abilities.
Harris et al., 2015 [38]	Project Viva (N=1,109)	Mean age: 8 years	Retrospective cohort/cross-sectional	- None of the exposure indicators (residential traffic density, BC between birth and age 6, $PM_{2.5}$ between birth and age 6, BC year before examination, $PM_{2.5}$ year before examination) negatively associated with verbal IQ, non-verbal IQ, visual motor abilities, design memory, or picture memory, adjusting for a broad set of potential confounders.

Reference	Study group	Age	Study design	Results
Kicinski et al., 2015 [39], Chapter 4	Flemish adolescents (N=606)	Mean age: 14.9 years	Cross-sectional	- A factor based on a urinary metabolite of benzene (*t,t*-MA-U) and the amount of contact with traffic preceding the neurobehavioral examination negatively associated with sustained attention, adjusting for gender, age, smoking, passive smoking, level of education of the mother, socioeconomic status, time of the day, and day of the week. - Associations with short-term memory and manual motor speed not statistically significant.
Kicinski et al., Chapter 5	Non-smoking Flemish adolescents (N=931)	Mean age: 14.9 years	Cross-sectional	- *t,t*-MA-U inversely associated with sustained attention, adjusting for gender, age, passive smoking, the education level of the parents, family income, time of the day, day of the week, and the logarithm of blood lead level.
Kicinski et al., Chapter 6	Primary school children (N=334)	Mean age: 10.2 years	Panel (3 measurements per child)	- Acute changes in air pollution level (UFP, $PM_{2.5}$, PM_{10}, and NO_2) not associated with attention, short-term memory, or visual information processing speed. - Past-year and lifetime PM exposure adversely associated with attention in the analysis not accounting for multiple testing and not taking a potential confounder school into account.
Siddique et al., 2011 [40]	Children from Delhi (N=969) and controls from a rural area (N=850) matched for age	9-17 years	Cross-sectional	- PM_{10} concentrations associated with a higher ADHD prevalence, controlling for gender, age, socioeconomic status, and BMI.
Suglia et al., 2008 [41]	A sample from a birth cohort from Boston (N=202)	Mean age: 9.7 years	Retrospective cohort	- Lifetime residential black carbon exposure associated with the Visual and General (but not with Verbal or Learning) scales of the Wide Range Assessment of Memory and Learning and with the Matrices and Composite (but not Vocabulary) scales of the Kaufman Brief Intelligence Test, adjusting for age, gender, primary language spoken at home, mother's education, in-utero tobacco smoke, secondhand smoke, birth weight, and blood lead level.
Sunyer et al., 2015 [42]	Children from Barcelona (N=2,715)	7-10 years	Prospective cohort (4 measurements per child)	EC, NO_2, and UFP concentrations at locations of the schools attended by children associated with a smaller improvement during a period of 12 months of working memory and inattentiveness, adjusting for age, sex, maternal education, residential neighborhood socioeconomic status, and air pollution exposure at home.

Reference	Study group	Age	Study design	Results
Van Kampen et al., 2012 [43]	Children from the RANCH project attending schools around Schiphol-Amsterdam Airport (N=553)	9-11 years	Cross-sectional	- Mean annual school concentrations of NO_2 negatively associated with memory (measured by the Digit Memory Span Test from the Neurobehavioral Evaluation System battery), adjusting for gender, age, employment status, crowding, home ownership, mother's education, long-standing illness, main language spoken at home, parental support, double glazing at home, road- and air traffic noise. - No associations with performance in the Simple Reaction Time, Switching Attention, Hand-Eye Coordination, or Symbol Digit Substitution tests. - No associations of residential NO_2 with any neurobehavioral outcomes.
Wang et al. 2009 [44]	Children from Quanzhou, China, attending two schools: one located in a polluted and another one located in a clean area (N=928)	8-10 years	Cross-sectional	- Children from the school located in a clean area performed better in the Continuous Performance Test (from the Neurobehavioral Evaluation System battery) and in the Digit Symbol, Pursuit Aiming, and Sign Register tests (from the Jinyi Psychomotor Test Battery). - No differences found in performance in the Line Discrimination, Visual Retention, or Simple Reaction Time tests (Neurobehavioral Evaluation System battery) or in performance in the Digit Erase Test (Jinyi Psychomotor Test Battery).

Table 2. Perinatal air pollution exposure and neurodevelopment.

Reference	Study group	Age	Study design	Results
Edwards et al., 2010 [45]	A birth cohort from Cracow, Poland (N=214)	5 years	Prospective cohort	- Prenatal PAH's exposure assessed by personal air monitoring in the mother associated with impaired intelligence (assessed using the Raven Progressive Matrices Test), adjusting for prenatal environmental tobacco smoke, gender, and maternal education.
Guxens et al., 2012 [46]	A birth cohort from Spain (N=1,889)	14 months	Retrospective cohort	- Mean residential NO_2 and benzene concentrations during the pregnancy not associated with mental development assessed by the Bayley Scales of Infant Development.
Guxens et al., 2014 [47]	Children from European cohorts GENERATION R, DUISBURG, EDEN, GASPII, RHEA, and INMA (N=9,482)	1-6 years	Retrospective cohort	- Mean residential NO_2 concentrations during the pregnancy associated with psychomotor development in early childhood. - No associations for the five other exposure indicators (NO_x, PM_{10}, $PM_{2.5}$, PM_{coarse}). - No associations with cognitive development or language development.

Reference	Study group	Age	Study design	Results
Harris et al., 2015 [38]	Project Viva (N=1,109)	Mean age: 8 years	Retrospective cohort	- Non-verbal IQ, design memory, picture memory but not verbal IQ or visual motor abilities associated with residential traffic density at birth. - Residential distance to nearest major road at birth smaller than 50 meters associated with lower non-verbal IQ (reference: ≥200 meters), adjusting for a broad set of potential confounders. - BC and $PM_{2.5}$ in the third trimester not associated with cognition.
Jedrychowski et al., 2015 [48]	A subsample from the birth cohort in Cracow (N=170)	7 years	Prospective cohort	Higher PAH–DNA adducts in cord blood associated with higher depressed verbal IQ score, adjusting for birth season, indoor PAH exposure, maternal education, gender, parity, and breastfeeding.
Kim et al., 2014 [49]	Children from the MOCEH study (N=520)	Age at follow-ups: 6, 12, and 24 months	Prospective cohort	- Average residential PM_{10} concentrations during pregnancy associated with mental development and psychomotor development, adjusting for sex, birth weight, maternal age, gestational age, maternal education, and family income. - Average residential NO_2 concentrations during pregnancy associated with psychomotor development but not with mental development.
Newman et al., 2013 [50]	A subsample from the Cincinnati Childhood Allergy and Air Pollution Study (N=576)	7 years	Retrospective cohort	- The average residential concentration of elemental carbon attributed to traffic during the first year of life associated with hyperactivity measured by the Behavioral Assessment System for Children, Parent Rating Scale, adjusting for gender, environmental tobacco smoke exposure in the first year of life, and maternal education. - No associations with other ADHD related outcomes (attention problems, aggression, conduct problems or atypicality).
Perera et al., 2006 [51]	Children of African-American and Dominican mothers from New York (N=183)	Age at follow-ups: 1, 2, and 3 years	Prospective cohort	- Prenatal PAH's exposure above the third quartile measured by personal air monitoring of the mother associated with a lower Mental Development Index of the Bayley Scale of Infant Development at age 3, adjusting for ethnicity, gender, gestational age, and home environment. - No association with Psychomotor Development Index or behavioral problems assessed by the Child Behavior Checklist. - No associations at age 1 or 2.
Perera et al., 2009 [52]	Children of African-American and Dominican mothers from New York (N=249)	5 years	Prospective cohort	- Prenatal PAH's exposure above the median measured by personal air monitoring in the mother associated with full-scale IQ and verbal IQ of the Wechsler Preschool and Primary Scale of Intelligence-Revised, adjusting for ethnicity, gender, prenatal environmental tobacco smoke, maternal education, maternal IQ, and quality of the home caretaking environment. - No associations with performance IQ.

Reference	Study group	Age	Study design	Results
Perera et al., 2011 [53]	Children of African-American and Dominican mothers from New York (N=215)	Mean age at follow-ups: 5 years and 7 years	Retrospective cohort	- Higher PAH–DNA adducts in cord blood associated with higher symptom scores of Anxious/Depressed and Anxiety Problems at 5 years and Attention Problems at 5 and 7 years (assessed with the Child Behavior Checklist), adjusting for prenatal environmental tobacco smoke, gender, gestational age, maternal IQ, quality of the home caretaking environment, maternal education, ethnicity, prenatal demoralization, age at assessment, and heating season. - Associations between the exposure and the evolution of scores over time not reported.
Perera et al., 2012 [54]	Children of African-American and Dominican mothers from New York (N=253)	7 years	Prospective cohort	- Prenatal PAH's exposure associated with higher symptom scores of Anxious/Depressed, Attention Problems, and Anxiety Problems (the Child Behavior Checklist), adjusting for prenatal environmental tobacco smoke, sex of child, gestational age, maternal IQ, quality of the home caretaking environment, maternal education, ethnicity, prenatal demoralization, age at assessment, and heating season.
Roberts et al., 2013 [55]	Children of participants from the Nurses' Health Study (325 cases of autism and 22,101 controls)	3-18 years	Case-control	- Residential annual average concentrations of diesel PM at the year of birth associated with an increased risk of autism, adjusting for maternal age at birth, year of birth, maternal parents' education, census tract median income, census tract percent college educated, and hazardous air pollutant model year.
Volk et al., 2011 [56]	Cases (N=304) and controls (N=259) matched for gender, age, and area from the Childhood Autism Risks from Genetics and the Environment (CHARGE) study in California	24-60 months	Case-control	- Living <309 m (10th percentile) from freeway at the time of delivery associated with a higher risk of autism (reference: >1.419 m), OR=1.86, 95% CI: 1.04-3.45, adjusting for gender, ethnicity, maximum education of parents, maternal age, and maternal smoking during pregnancy. - Distance to major road not associated with autism.
Volk et al., 2013 [57]	Cases and controls matched for gender, age, and area from the CHARGE study in California (N=524)	24-60 months	Case-control	- Higher risk of autism for the highest quartile of the average residential traffic-related air pollution concentrations (estimated using nitrogen oxides), PM_{10}, $PM_{2.5}$, and NO_2 during the first year of life, pregnancy, the first trimester, second trimester, and third trimester (reference: the lowest quartile), adjusting for gender, ethnicity, maximum education of parents, maternal age, and maternal smoking during pregnancy.

Public health implications

The public health significance of the effect size that we observed in the study of the association between traffic exposure and neurobehavioral performance of adolescents can be illustrated by comparing it with the effect of parental education. A 10-fold increase in the level of *t,t*-MA-U was associated with a 0.17 SD (95% CI: -0.31 to -0.04) decrease in sustained attention. The estimated difference between the mean sustained attention of children having at least one parent with a university or college diploma and children with both parents without a high school diploma equaled 0.37 SD (95% CI: -0.55 to -0.18). A decrease of 0.17 SD in sustained attention for a ten-fold increase in the level of *t,t*-MA-U does not seem very important in terms of the amount of distress caused to an individual. However, such effect is important at the population level, because exposure to traffic-related air pollution affects the whole population [58,59]. Moreover, subtle neurobehavioral deficits may, with prolonged exposure, progress to more severe effects [60-62].

LIMITATIONS OF EPIDEMIOLOGICAL RESEARCH ON THE NEUROBEHAVIORAL EFFECTS OF ENVIRONMENTAL POLLUTION

The possibility of reverse causation is an important limitation of observational studies [63]. In environmental epidemiology, reverse causation refers to a situation when the level of exposure is affected by outcome. Reverse causation was not a potential source of bias in the study of the effect of acute changes in the air pollution level on neurobehavioral performance of primary school children. The within-person changes in neurobehavioral capabilities do not affect the within-person changes of the level of ambient air pollution at schools. However, reverse causation cannot be excluded in the other studies of this PhD project. We cannot rule out that the motor function is the causing factor in the association between manual motor speed and PBDEs exposure in adolescents. For example, children with poorer motor skills may choose hobbies involving a higher level of exposure (e.g., those requiring to spend time at home). It is also possible that the cognitive skills of an adolescent (and his family) affect the level of copper exposure. Although it is not clear how sustained attention could have an effect on the level of traffic-related air pollution exposure, we cannot rule out such a possibility.

Another limitation of observational studies is the possibility of bias due to confounding. Confounding occurs when exposure would remain associated with outcome even if all exposure effects were removed [64,65]. Possible sources of confounding include genetic, demographic, socioeconomic, and

lifestyle characteristics of the participants and time-related factors (e.g., time of the day). Also environmental contamination by chemicals different than those considered as risk factors can bias study results. We took several precautions in order to limit the risk of confounding. In all analyses, we included a set of potential confounders that we identified based on the literature. Because socioeconomic status is an important confounder of the association between environmental pollution and human health, we measured it with multiple indicators including parental education level, family income, and occupation of the parents. We performed the analysis of the association between traffic-related air pollution exposure and neurobehavioral performance of adolescents excluding smokers since smoking strongly affects the level of *t,t*-MA-U, which we used as exposure indicator. In the study of the acute effects of air pollution in primary school children, we used a study design that excluded the risk of confounding of person-related characteristics. However, despite these precautions, we cannot exclude confounding due to factors that we failed to account for or that we measured imprecisely.

Experimental *in vitro* and animal studies are completely free of the risks of reverse causation and confounding. However, drawing conclusions about humans based on *in vitro* or animal models involves several uncertainties including those related to cross-species extrapolation and differences in exposure between experimental models and humans. Epidemiological research directly evaluates health effects in the population of interest at relevant exposure levels. Therefore, despite their limitations, observational human studies are an important source of information for environmental risk assessment [66-68]. Both in the case of effects of traffic-related air pollution on sustained attention and effects of PBDEs on the motor function, experimental evidence using animal models supports our findings.

Error in the measurement of exposure is another potential source of bias. It may have many possible causes, including imprecision of laboratory techniques or the use of a measurement at a single point in time or space when the total exposure is of interest. Common consequences of error in the measurement of exposure include a bias in the estimate of the association between exposure and outcome and a decrease of the statistical power [69]. When the measurement error is not systematic (i.e., when exposure is measured without a systematic bias), it leads to an attenuation of regression coefficients [69,70]. Therefore, under this assumption, error in the measurement of exposure does not increase the risk of a false-positive result.

Error in the measurement of outcomes represents another problem in epidemiological research of the neurobehavioral effects of environmental pollution. Neurobehavioral domains such as sustained attention or short-term memory are unavoidably measured with an error. One reason for this is that neurobehavioral performance may be affected by factors not related to neurobehavioral skills, such as the motivation of the study participant.

Moreover, different neurobehavioral domains have an impact on performance measured by a specific parameter. For example, the mean reaction time in the Continuous Performance Test, a measure of sustained attention, is also affected by the motor skills [71]. Error in the measurement of outcome increases the uncertainty about the estimates of the effect of exposure and, consequently, decreases the study power [72]. In this PhD project, we used several indicators for each neurobehavioral domain studied in order to improve the outcome assessment.

A small sample size is a common problem in biomedical studies. It may result in an insufficient power to detect an effect of exposure. Additionally, what is less appreciated, a small sample size increases the probability that a research finding that is claimed to be statistically significant is actually false [73]. One way to deal with the problem of an insufficient sample size in an individual research project is to combine results from multiple studies in a meta-analysis. This PhD project contributed to the international efforts of gathering data for meta-analyses of the associations between environmental pollution and neurobehavioral outcomes in primary school children and adolescents.

CONCLUSIONS

In this PhD project, we investigated the associations between environmental pollution and neurobehavioral performance in primary school children and adolescents. In adolescents, we concentrated on three groups of pollutants: brominated flame retardants, toxic metals, and traffic-related air pollution. In primary school children, we considered the effects of acute changes in the air pollution level and of the long-term residential air pollution exposure.

Consistent with animal studies, we found evidence of an inverse association between exposure to PBDEs and the manual motor speed in adolescents, supporting the recent policies to limit the use of PBDEs. Neurobehavioral performance was not associated with exposure to HBCD and TBBPA, brominated flame retardants that are still in use today.

Blood copper was inversely associated with sustained attention and short-term memory in a population of adolescent girls with normal copper levels, adding to the preliminary evidence of neurotoxic effects of a low-level copper exposure and suggesting that these effects should be extensively investigated by future studies. We found no evidence that other toxic metals were associated with neurobehavioral performance of adolescents at the current exposure levels.

Traffic exposure as indicated by *t,t*-MA, a urinary metabolite of benzene, and the estimate of the amount of contact with traffic was inversely associated with sustained attention in a study of 606 adolescents. This result adds to the

growing body of evidence that traffic-related air pollution exposure negatively affects the neurobehavioral function. Further support for an association between traffic exposure and attention was found in the analysis based on an extended study group of 931 non-smoking adolescents. In this analysis, a 10-fold increase of *t,t*-MA-U was associated with a 0.17 SD decrease in sustained attention (95% CI: -0.31 to -0.04).

In primary school children, we found no evidence of an association between acute changes of the air pollution level and attention, short-term memory, or visual information processing speed. To our best knowledge, this is the only study to investigate the neurobehavioral effects of a short-term air pollution exposure to date. Consistent with previous studies, we found some indication of a negative association between long-term residential particulate matter exposure and attention. The results of our study may reflect that a long-term air pollution exposure is more relevant for neurobehavioral performance than acute changes in the air pollution level.

Despite their limitations, observational human studies play an important role in human health risk assessment. The studies performed as a part of this PhD project contribute to the international research investigating the neurotoxic effects of environmental pollutants at the current exposure levels.

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Appendices

CURRICULUM VITAE

Michal Kicinski was born in 1984 in Poland. In 2007, he studied cognitive psychology at the University of Maastricht in the Netherlands. In 2008, he finished his Master's degree in Psychology at the University of Warsaw in Poland. In 2011, he obtained a Master's degree in statistics with a specialization in biostatistics at the University of Leuven in Belgium. In 2011, he worked as a researcher at the University of Ghent in Belgium. Since October 2011, he is a PhD fellow of the Research Foundation Flanders (FWO) and conducts his PhD research project at the Centre of Environmental Sciences at the University of Hasselt. In July 2015, he was an invited speaker at the Fifteenth Biennial Meeting of the International Neurotoxicology Association meeting in Montreal, Canada.

LIST OF PUBLICATIONS

Articles in peer-reviewed journals

1. Kicinski M, Vrijens J, Vermier G, Hond ED, Schoeters G, Nelen V, Bruckers L, Sioen I, Baeyens W, Van Larebeke N, Viaene MK, Nawrot TS: Neurobehavioral function and low-level metal exposure in adolescents. *Int J Hyg Environ Health* 2015, 218:139-146.
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