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COSEMANS, Charlotte; Van Larebeke, Nicolas; JANSSEN, Bram; MARTENS, Dries; Baeyens, Willy; BRUCKERS, Liesbeth; Den Hond, Elly; Coertjens, Dries; Nelen, Vera; Schoeters, Greet; Hoppe, Hans-Wolfgang; WOLFS, Esther; SMEETS, Karen; NAWROT, Tim & PLUSQUIN, Michelle (2022) Glyphosate and AMPA exposure in relation to markers of biological aging in an adult population-based study. In: International journal of hygiene and environmental health (Print), 240 (Art N° 113895).

DOI: 10.1016/j.ijheh.2021.113895
Handle: http://hdl.handle.net/1942/36224

Glyphosate and AMPA exposure in relation to markers of biological aging in an adult population-based study

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ABSTRACT

BACKGROUND/AIM: Glyphosate, a broad-spectrum herbicide, and its main metabolite aminomethylphosphonic acid (AMPA) are persistent in the environment. Studies showed associations between glyphosate or AMPA exposure and several adverse cellular processes, including metabolic alterations and oxidative stress.

OBJECTIVE: To determine the association between glyphosate and AMPA exposure and biomarkers of biological aging.

METHODS: We examined glyphosate and AMPA exposure, mtDNA content and leukocyte telomere length in 181 adults, included in the third cycle of the Flemish Environment and Health Study (FLEHSIII). DNA was isolated from leukocytes and the relative mtDNA content and telomere length were determined using qPCR. Urinary glyphosate and AMPA concentrations were measured by Gas Chromatography-Tandem Mass Spectrometry (GC-MS-MS). We used multiple linear regression models to associate mtDNA content and leukocyte telomere length with glyphosate or AMPA exposure while adjusting for confounding variables.

RESULTS: A doubling in urinary AMPA concentration was associated with 5.19% (95% CI: 0.49 to 10.11; p = 0.03) longer leukocyte telomere length, while no association was observed with urinary glyphosate

concentration. No association between mtDNA content and urinary glyphosate nor AMPA levels was observed.

CONCLUSIONS: This study showed that AMPA exposure may be associated with telomere biology in adults.

KEYWORDS: Glyphosate, AMPA, telomere length, mtDNA content

ABBREVIATIONS

AHS	Agricultural Health Study
AMPA	Aminomethylphosphonic acid
BUND	Bund für Umwelt und Naturschutz Deutschland
EPSPS	5-enolpyruvylshikimate 3-phosphate synthase
FLEHS	Flemish Environment and Health Study
G-EQUAS	German External Quality Assessment Scheme
GC-MS-MS	Gas Chromatography-Tandem Mass Spectrometry
IARC	International Agency for Research on Cancer
IQR	Interquartile range (25th – 75th percentile)
LLOQ	Lower limit of quantitation
NHANES	National Health and Nutrition Examination Survey
PCB	Polychlorinated biphenyl
POP	Persistent organic pollutant
ROS	Reactive oxygen species

INTRODUCTION

Glyphosate (N-[phosphonomethyl]-glycine) is a broad-spectrum, non-selective herbicide used in agricultural formulations worldwide. Glyphosate is the active ingredient in the commercial formulation Roundup®, which was first sold by Monsanto in 1974. From 1974 to 2014, agricultural use of glyphosate rose 300-fold, while non-agricultural use increased 43-fold. Following the introduction of genetically modified herbicide-tolerant crops in 1996, agricultural applications of glyphosate boomed (Benbrook, 2016). Glyphosate's main degradation metabolite is AMPA, which is formed by microbial degradation in the soil (Agostini et al., 2020). Both glyphosate and AMPA are known to persist in the environment and can be found in house dust (Curwin et al., 2005), soil, air, surface water, and groundwater (Bai and Ogbourne, 2016; IARC, 2017). Humans are mainly indirectly exposed through contaminated food, posing a potential long-term threat to human health. Glyphosate is poorly metabolised – and exclusively to AMPA – in humans and is mainly excreted in urine (Agostini et al., 2020). A recent study (Connolly et al., 2020) emphasized the importance of human biomonitoring studies as a measure of internal exposure. In their

comprehensive review, they reported 21 studies using human biomonitoring to assess urinary glyphosate and/or AMPA concentrations and found glyphosate to be omnipresent in the environment. Furthermore, other studies confirmed that urinary glyphosate levels are good exposure markers, since glyphosate does not accumulate and is insufficiently metabolised in humans (Agostini et al., 2020; Zoller et al., 2020). The recent finding that glyphosate's excretion fraction in urine is 1% (Zoller et al., 2020) suggested that the back-calculated oral dose would be 20 times higher than previously assumed (Connolly et al., 2020).

Several epidemiological studies reported associations between glyphosate exposure and different health disorders, including cancer (Leon et al., 2019), respiratory diseases (Hoppin et al., 2008), chronic kidney disease (Gunatilake et al., 2019), and neurological diseases (von Ehrenstein et al., 2019), as well as metabolic alterations and oxidative stress (Agostini et al., 2020; Meftaul et al., 2020). The toxicity of AMPA is ambiguous as it was reported to be similar or less compared to glyphosate (Moore et al., 2012) and in contrast, due to its longer persistence in the environment, some studies demonstrated that environmental AMPA toxicity was higher than that of glyphosate (Daouk et al., 2013; Guilherme et al., 2014). Although several studies focused on the effect of glyphosate and AMPA exposure on human health (Agostini et al., 2020), including disturbance of the oxidative balance and DNA damage, the association of glyphosate or AMPA exposure on biomarkers of aging such as mitochondrial DNA (mtDNA) content and telomere length is poorly investigated.

Mitochondria are intracellular organelles responsible for energy production via oxidative phosphorylation. In addition, they also play a role in apoptosis and reactive oxygen species (ROS) production (Martens and Nawrot, 2016). Mitochondria are especially vulnerable to oxidative damage since they are the main intracellular source as well as targets of ROS. mtDNA lacks several protective structures, like chromatin, histones, and DNA repair mechanisms, resulting in a high mutation rate (Janssen et al., 2012). This high mutation rate was associated with changes in mtDNA content (Kauppila et al., 2017; Vriens et al., 2019). Several studies reported a lower mtDNA content with chronological aging (Chistiakov et al., 2014; Knez et al., 2016; Seo et al., 2010). Telomeres are ribonucleoprotein structures consisting of 5'-TTAGGG-3' tandem repeats positioned at the end of chromosomes, forming protection from degradation, ensuring genome stability, and preventing loss of genetic information (Blackburn, 1991). During each mitotic cycle, telomeres shorten due to the inability of DNA polymerase to preserve the length of the 3' overhang, which is known as the end-replication problem (Fragkiadaki et al., 2020). Telomere length is maintained by telomerase, which is an enzyme responsible for adding the 5'-TTAGGG-3' tandem repeats to the ends of chromosomes, and is mainly active in germ cells, stem cells, and immortalized cells (Martens and Nawrot,

2016). Both mtDNA content and telomere length can be affected by various lifestyle factors and are considered to be biomarkers of biological aging. Accumulating evidence linked these biomarkers to age-related diseases (Chistiakov et al., 2014; Sahin and DePinho, 2012; Seo et al., 2010). Exposure to environmental pollutants, like glyphosate and AMPA, may be associated with the onset of age-related diseases (Vriens et al., 2019). Therefore, in this study we investigated the association between glyphosate and AMPA exposure and markers of biological aging in a study population of the Flemish Environment and Health Study (FLEHS).

MATERIALS AND METHODS

STUDY POPULATION

This study was part of the third cycle of the FLEHS, which collects data on human environmental exposures as well as human biological samples in Flanders, Belgium. Inclusion criteria were: i) age between 50-65 years, ii) living at least 10 years in Flanders, iii) not having active cancer treatment or renal pathologies, and iv) being able to fill out an extensive questionnaire in Dutch. In total, 209 participants were included in this study. More details of the recruiting procedure are available in the supplemental information. After excluding 28 subjects due to missing data (telomere length: n = 11, mtDNA content: n = 9, AMPA concentration: n = 2, other covariates: n = 6), data were analysed for 181 adults.

The medical ethical committee of the University Hospital of Antwerp and the University of Antwerp approved the study (Belgian registration number: B300201419834). Informed consent was obtained from each individual for study participation. This study has been carried out according to the Helsinki declaration.

DATA AND SAMPLE COLLECTION

Data obtained by self-administered questionnaires included information on lifestyle factors and socioeconomic status. Smoking status was defined as never smoked, former smoker or current smoker and alcohol consumption as never, less than monthly, less than weekly or weekly consumption. Socioeconomic status was based on the highest household educational level, coded low (maximum lower grade of secondary school), middle (secondary school) or high (college or university). BMI was calculated as kg/m^2. To correct for urinary concentration, glyphosate and AMPA concentrations were normalized to the urine specific gravity using the following formula: exposure marker*[(1.024 – 1)/(urine specific gravity – 1)]. Urine samples were collected in metal-free polyethylene containers. Blood samples were collected

in EDTA Vacutainer Blood Collection Tubes and immediately centrifuged for serum collection. Samples were stored within 24 h at -80°C until further use.

HANDLING OF MEASUREMENTS BELOW QUANTITATION LIMITS

Exposure measurements with observations below the lower limit of quantitation (LLOQ) were treated with Censored Likelihood Multiple Imputation by using the *lodi* package in R. A range of 20 – 100 imputations was suggested, since a high number of imputations may cause simulation error (van Buuren, 2018). Therefore, we opted to carry out 20 imputations. In addition, to test the robustness of our results, the AMPA concentration was 15% trimmed to account for the measurements below the LLOQ, according to the Guidelines for Data Quality Assessment (version QA00) of the Environmental Protection Agency (EPA). Also, both single imputation by fixed value (i.e., LLOQ/2) and random imputation using values between 0 and LLOQ were investigated. Glyphosate and AMPA concentrations were also investigated as binary variables, coded 0 when below the LLOQ and 1 when above the LLOQ.

MEASUREMENT OF MITOCHONDRIAL DNA CONTENT AND TELOMERE LENGTH

Mitochondrial DNA (mtDNA) content (Janssen et al., 2012) and telomere length (Martens et al., 2016) were measured as described elsewhere. Briefly, DNA was isolated from the buffy coat, containing leukocytes, using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). The relative mtDNA content was measured by determining the ratio of two mitochondrial gene copy numbers (MT-ND1 and MTF3212/R3319) to a single copy nuclear control gene (RPLP0). Relative telomere length was determined by the ratio of the telomere sequence to the RPLP0 control gene, proportional to the mean telomere length of the study population. Both mtDNA content and telomere length were measured using the 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). The full protocol is available in the supplemental information.

MEASUREMENT OF GLYPHOSATE AND AMPA

Samples were shipped on dry ice and stored at -18°C until further use. Urinary glyphosate and AMPA concentrations were measured according to the procedure described elsewhere (Alferness and Iwata, 1994) with some modifications (Hoppe, 2013). Briefly, 100 µL urine and 50 µL of the working solution of the internal standard were transferred to screw-capped glass tubes containing 1 mL of acetonitrile. After evaporation to dryness in a vacuum centrifuge for derivatization, 0.5 mL of 2,2,2-trifluoroethanol and 1 mL of freezing cold (-40°C) trifluoroacetic anhydride were added to the residue. The mixture was heated to 85°C for 1 h. After cooling, the solution was cautiously evaporated at 80-85°C without a stream of air or

nitrogen. After cooling, the oily residue was dissolved in 200 µL of acetonitrile and measured by Gas Chromatography-Tandem Mass Spectrometry (GC-MS-MS). The urine samples were distributed across 11 runs. Each run included seven calibration standards, a blank and a calibration verification at 1 µg/L. Two quality control samples with known glyphosate/AMPA spiked concentration and a real-life urine sample of an exposed person were included. The LLOQ for both glyphosate and AMPA was based on the lowest calibration standard of 0.1 µg/L. Details of GC-MS-MS analysis and method validation were reported elsewhere (Conrad et al., 2017). With the presented method, the contracted laboratory successfully participated in the recent three rounds of the German External Quality Assessment Scheme (G-EQUAS) for glyphosate measurements in urine. The target levels of the control material ranged from 0.42 to 3.73 µg/L and the analysed concentrations deviated from 1.1 to 7.1%, confirming the accuracy of the method. Reference values can be obtained at https://app.g-equas.de/web/info (G-EQUAS 64-66). Currently, G-EQUAS only includes glyphosate, not AMPA.

STATISTICAL ANALYSIS

Data management and statistical analysis were done using RStudio software (version 1.1.463), R version 3.6.3. Glyphosate and AMPA concentration, as well as telomere length and mtDNA content were log10 transformed to normalize their distribution. The association between glyphosate and AMPA in urine and markers of biological aging (i.e., mtDNA content and telomere length) was explored using multiple linear regression models adjusted for the following *a priori* selected covariates: sex, age, BMI, smoking status, alcohol consumption, socioeconomic status, season of sampling, and urine specific gravity. Models exploring the association between glyphosate and AMPA exposure and mtDNA content were additionally adjusted for platelet count (amount/µL). Since DNA was extracted from leukocytes, we additionally adjusted for leukocyte count (amount/µL) in sensitivity analyses. Glyphosate exposure has been linked with chronic kidney disease, hence the additional adjustment for cystatin C (mg/L) and alfa-1-microglobulin (mg/L) in sensitivity analyses. For descriptive purposes, continuous variables (i.e., age, BMI, urine specific gravity, mtDNA content, and telomere length) were presented as means ± standard deviation (SD) and categorical variables (i.e., sex, smoking status, alcohol consumption, socioeconomic status, and season) as numbers (frequency in percentage). All reported *p*-values were considered significant when $p < 0.05$. Estimates were provided as the % difference (95% CI) of mtDNA content or telomere length from a doubling in glyphosate or AMPA concentration.

RESULTS

POPULATION CHARACTERISTICS

The 181 participating adults (51.9% women) were 58.2 ± 4.0 (SD) years old and had a mean BMI of 25.5 ± 4.2 kg/m². The majority never smoked (45.9%), while 40.9% were former smokers. Most of the participants consumed alcohol on a weekly basis (64.1%) and had a high socioeconomic status based on the highest obtained diploma in the household (55.8%) (**Table 1**). 57.5% of glyphosate and 41.4% of AMPA measurements were below the LLOQ. The geometric mean for the urinary glyphosate and AMPA concentration was 0.11 µg/L (IQR: 0.05 – 0.20) and 0.16 µg/L (IQR: 0.08 – 0.33), respectively (**Figure 1**). The raw analytical data for the urinary glyphosate and AMPA concentrations are shown in **Table 2**. Urinary glyphosate and AMPA concentrations were positively correlated ($R = 0.55$; $p < 0.0001$), as well as leukocyte mtDNA content and telomere length ($R = 0.56$; $p < 0.0001$) (**Supplementary Figure 1**). The population characteristics of the trimmed population are shown in **Supplementary Table 1**.

Table 1: Study population characteristics (n = 181).

Characteristic	Mean ± SD or *n* (%)
Age (years)	58.2 ± 4.0
Sex (female)	94 (51.9)
BMI	25.5 ± 4.2
Smoking status	
Never	83 (45.8)
Former smoker	74 (40.9)
Current smoker	24 (13.3)
Alcohol consumption	
Never	16 (8.8)
< Monthly	34 (18.8)
< Weekly	15 (8.3)
Weekly	116 (64.1)
Socioeconomic status	
Low	38 (21.0)
Middle	42 (23.2)
High	101 (55.8)
Season of sampling	
Winter	-

Spring	36 (19.9)
Summer	69 (38.1)
Autumn	76 (42.0)
Urine specific gravity	1.0 ± 0.01
Leukocyte count	6970 ± 1630

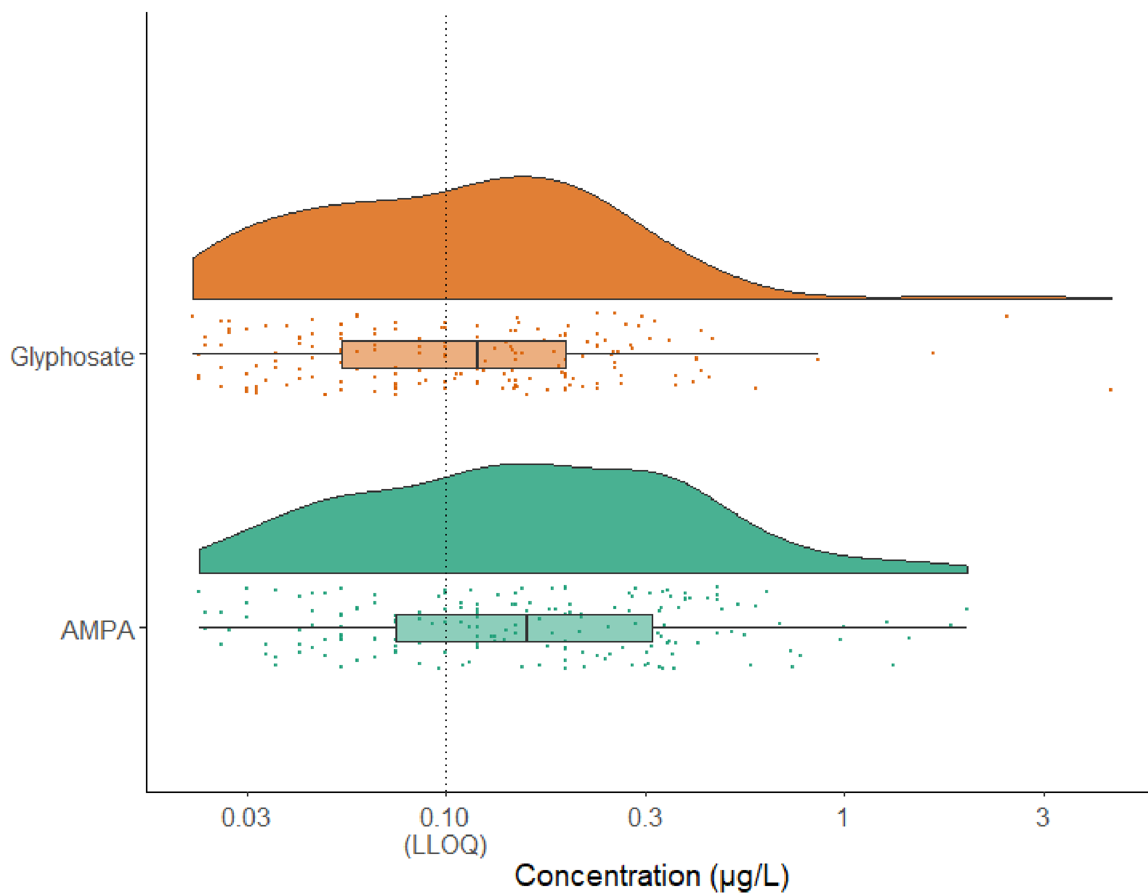


Figure 1: Raincloud plot for glyphosate and AMPA concentration (adjusted for urine specific gravity) (n = 181). The geometric mean for the urinary glyphosate and AMPA concentration was 0.11 µg/L (IQR: 0.05 – 0.20) and 0.16 µg/L (IQR: 0.08 – 0.33), respectively. The x-axis shows log10 transformed values. The dotted line represents the LLOQ.

Table 2: Raw analytical data for the urinary glyphosate and AMPA concentrations. Data are shown for the 50th, 75th, 90th, and 95th percentile, as well as the maximum concentration.

	50th	**75th**	**90th**	**95th**	**Max**
Glyphosate (µg/L)	<LLOQ	0.13	0.22	0.31	3.72

AMPA (µg/L)	0.10	0.18	0.34	0.40	1.50

ASSOCIATION OF URINARY GLYPHOSATE OR AMPA CONCENTRATION AND MARKERS OF BIOLOGICAL AGING

After adjustment for sex, age, BMI, smoking status, alcohol consumption, socioeconomic status, season of sampling, and urine specific gravity, a doubling in glyphosate concentration was not associated with leukocyte telomere length (p = 0.15), however the urinary concentration of the glyphosate metabolite AMPA was associated with a 5.19% (95% CI: 0.49 to 10.11; p = 0.03) longer leukocyte telomere length (**Table 3**). No associations were found between AMPA or glyphosate concentrations and mtDNA content.

Table 3: The association between glyphosate and AMPA exposure and markers of biological aging (n = 181). Estimates were provided as the % difference (95% CI) of telomere length or mtDNA content from a doubling in glyphosate or AMPA concentration. Models were adjusted for sex, age, BMI, smoking status, alcohol consumption, socioeconomic status, season, and urine specific gravity. mtDNA content has been additionally adjusted for platelet count.

	% difference (95% CI)	***p*-value**
Telomere length		
Glyphosate	3.31 (-1.17 to 8.07)	0.15
AMPA	5.19 (0.49 to 10.11)	0.03
mtDNA content		
Glyphosate	2.38 (-4.34 to 9.51)	0.50
AMPA	4.32 (-3.07 to 12.19)	0.26

In sensitivity analyses (**Table 4**) for the association between AMPA exposure and telomere length, additionally adjusting for leukocyte count and cystatin C and alfa-1-microglobulin did not affect the experimental outcome. Using the trimmed population, single imputation, and random imputation, the association between urinary AMPA concentration and telomere length remained significant (p = 0.01, p = 0.045, p = 0.046 respectively; **Supplementary Table 2**). Also, when using AMPA concentration as a binary variable, the results remained the same (p = 0.03; **Supplementary Table 3**).

Table 4: Sensitivity analysis for the association between AMPA concentration and telomere length (model 1). Estimates were provided as the % difference (95% CI) of telomere length from a doubling in AMPA concentration. Models were adjusted for sex, age, BMI, smoking status, alcohol consumption, socioeconomic status, season, and urine specific gravity.

	n	**% difference (95% CI)**	***p*-value**
Additionally adjusted for leukocyte count	181	0.91 (0.06 to 5.19)	0.047

Additionally adjusted for cystatin C and alfa-1-microglobulin	179	1.82 (0.03 to 3.67)	0.046

DISCUSSION

The key point of our study is that urinary AMPA levels are associated with longer leukocyte telomere length in adults. To our knowledge, the present study is the first to investigate the association between glyphosate and AMPA exposure and biomarkers of aging in a population-based set-up.

Glyphosate and AMPA are persistent in the environment and present in food sources. Remarkably, only 21 human biomonitoring studies measuring glyphosate levels (and even fewer for AMPA levels) in urine have been published up till 2020 (Connolly et al., 2020). In our population-based study, urinary AMPA levels were significantly associated with leukocyte telomere length while this was not the case for urinary glyphosate levels. AMPA is more persistent in the environment than glyphosate, with an environmental half-life time of 151 days, ranging from 76 to 240 days depending on environmental conditions like temperature and soil moisture (Domínguez et al., 2016; Silva et al., 2018). In contrast, the biological half-life time of glyphosate in urine was within nine hours (Connolly et al., 2019; Zoller et al., 2020). The degradation of both glyphosate and AMPA is faster at warm and moist conditions (Bento et al., 2017). In a study investigating the presence of glyphosate and AMPA in agricultural top soils of the European Union, glyphosate and AMPA were present in 21% and 42% of the tested soil samples, respectively (Silva et al., 2018). Notably, it was suggested that the majority of urinary AMPA concentrations in human biomonitoring studies originated from exposure to AMPA itself (i.e., via food and water residues or from degradation of glyphosate in the soil) as opposed to the degradation of glyphosate in the body (Connolly et al., 2020). These findings might explain why we observed an association between telomere length and AMPA and not with glyphosate exposure. In our population, the mean urinary glyphosate and AMPA concentration was 0.11 µg/L and 0.16 µg/L, respectively. In 2013, the German Federal Institute for risk assessment (BfR) evaluated a Europe-wide investigation of glyphosate residues in human urine initiated by the German 'Bund für Umwelt und Naturschutz Deutschland' (BUND). They analysed 182 urine samples from 18 European countries to determine glyphosate and AMPA residues (BfR, 2013; Hoppe, 2013). The mean urinary glyphosate concentrations ranged from 0.09 µg/L in Bulgaria, Macedonia, and Switzerland to 0.82 µg/L in Malta, with an overall mean of 0.21 µg/L. The mean urinary AMPA concentration ranged from 0.08 µg/L in Macedonia and Switzerland to 0.40 µg/L in Malta, with an overall mean of 0.18 µg/L. Their results indicated that there are large regional and individual differences. Compared with their findings, the urinary concentrations for both glyphosate and AMPA were below average in our population.

In a retrospective analysis from 2001 – 2015 analysing 399 urine samples, 31.8% and 40.1% of the samples contained glyphosate and AMPA concentrations at or above the LLOQ, respectively. Both the highest measured concentrations for urinary glyphosate and AMPA were observed in 2013: 2.80 µg/L and 1.88 µg/L, respectively (Conrad et al., 2017). Up to 2020, the highest reported urinary concentration of non-occupational exposure to glyphosate was 9.4 µg/L (Curwin et al., 2007). In addition, occupational exposure to glyphosate showed a less than 10-fold increase in urinary glyphosate concentrations, with an average range from 1.35 µg/L to 3.2 µg/L (Connolly et al., 2020). In occupationally exposed workers in China, the maximum concentration of urinary glyphosate was 17.202 mg/L, while the highest measured urinary AMPA concentration was 2.73 mg/L (Zhang et al., 2020). .

Longer telomere length should provide prolonged cell survival, which in turn increases the chance of accumulation of cancer-causing mutations (Aviv et al., 2017; Noy, 2009; The Telomeres Mendelian Randomization, 2017). Several studies described the association between longer telomere length and an increased risk of different cancers such as non-Hodgkin lymphoma (Lan et al., 2009), lung cancer (Seow et al., 2014), and melanoma (Han et al., 2009). In addition, other studies reported an increase in telomere length in relation to an environmental exposure: i) the study of the National Health and Nutrition Examination Survey (NHANES) reported an age-independent association between exposures to polychlorinated biphenyls (PCBs) and longer leukocyte telomere length (Scinicariello and Buser, 2015), ii) another study (Shin et al., 2010) described higher leukocyte telomere length after exposure to low doses of persistent organic pollutants (POPs), and iii) urinary antimony and mercury exposure were positively associated with leukocyte telomere length (Vriens et al., 2019). Similar to glyphosate and AMPA, these chemicals are persistent in the environment and present in food sources. The Agricultural Health Study (AHS), a large prospective adult cohort study in the USA, published several studies investigating the association between glyphosate exposure and the risk of cancer. They found a relative risk of 2.6 for the development of multiple myeloma after exposure to glyphosate (De Roos et al., 2005). Another study (Flower et al., 2004) reported that the risk for cancer development was increased in children of glyphosate applicators, compared with children of the general population. However, the IARC (Humans, 2017) noted that both studies had limited power due to missing data on covariates or to study rare diseases like childhood cancer. In addition, case-control studies in Sweden (Eriksson et al., 2008), the USA (De Roos et al., 2003), and Canada (McDuffie et al., 2001) demonstrated an increased risk for the development of non-Hodgkin lymphoma. Using a mice model, a positive trend in the incidence of renal tubule carcinoma and hemangiosarcoma in male mice was reported following glyphosate exposure (Guyton et al., 2015; Humans, 2017). An almost twofold increase in the incidence of mammary gland tumours in female rats

who received glyphosate-based formulations through drinking-water was found, compared to control animals (Séralini et al., 2014). Since there was limited evidence in human studies and sufficient evidence in animal studies, the International Agency for Research on Cancer (IARC) classified glyphosate as probably carcinogenic to humans (Group 2A) (Humans, 2017). As enabling replicative immortality in cells is a hallmark of cancer development (Hanahan and Weinberg, 2011), our results suggest a possible underlying mechanism for tumour development related to AMPA exposure. Based on our findings, further investigating the association between telomeres and AMPA exposure in large cohorts is warranted. In addition, *in vitro* studies could further unravel the telomere-related mechanisms through which glyphosate and AMPA exert their potential carcinogenic effects.

Inflammation, adjusted detoxification mechanisms or oxidative stress are possible underlying mechanisms not only linked to telomere biology (Aubert and Lansdorp, 2008; Martens and Nawrot, 2016) but also to glyphosate exposure. Firstly, pollutants may induce acute inflammation (Pandey et al., 2019) in which immune cells proliferate rapidly (Dioni et al., 2011). Both naïve and memory B cells were reported to be capable of upregulating telomerase activity *in vitro* in response to activation signals (Weng et al., 1997). Thus leukocyte telomere length may not only be altered with aging, but also as a consequence of the activation and differentiation of immune cells (Hodes et al., 2002). Several studies using rodent models, investigated the effect of glyphosate exposure on the inflammation process. Another study (Kumar et al., 2014) demonstrated airway inflammation in mice after exposure to both farm air samples containing glyphosate and glyphosate alone. A dose-dependent adverse inflammatory effect after short-term exposure with Roundup® was reported in liver and adipose tissues of rats (Pandey et al., 2019). Using pregnant rats, changes in the expression of genes related to oxidative stress and inflammation after perinatal glyphosate-based herbicide exposure were reported (de Souza et al., 2019). Secondly, several studies described that glyphosate suppressed the activity of various cytochrome P450 (CYP450) enzymes (Samsel and Seneff, 2013). These enzymes are involved in the detoxification of xenobiotics and can produce carcinogenic metabolites (Pande et al., 2008). Another study (Eshkoor et al., 2013) reported that the *CYP1A2* gene polymorphism generated ROS, which causes DNA damage and carcinogenesis in cells. Thirdly, oxidative stress induced by glyphosate exposure in various tissues of rats has been broadly described (Cattani et al., 2014; Larsen et al., 2012; Turkmen et al., 2019). Glyphosate induced an increase in lipid peroxidation in pregnant rats (Beuret et al., 2005), as well as decreased glutathione levels in rats (Cattani et al., 2014) and enhanced H_2O_2 production in *C. elegans* (Bailey et al., 2018).

Recently, multiple studies investigated the toxicity of glyphosate using different human cell lines, both in healthy and tumour cells (Koller et al., 2012; Li et al., 2013; Mesnage et al., 2013). Specifically stem cells have been used for toxicity testing, for understanding cell proliferation and differentiation, and for investigating their role in the aging process (Kang and Trosko, 2010). Notably, one of the mechanisms responsible for cellular effects might be stem cell dysfunction due to telomere interference (Fragkiadaki et al., 2020; Ganguly et al., 2017).

This study has the following strengths and limitations. Our population-based study was part of FLEHS III and thus is representative for the middle-aged population living in Flanders. Missing data due to observations <LLOQ is a common obstacle in environmental epidemiological studies, by which our study was affected as AMPA had 41.4% and glyphosate 57.5% measurements <LLOQ. The IQR for both glyphosate and AMPA concentration is going below the LLOQ. We therefore performed four steps to treat the imputation of these observations. First, we followed the Guidelines for Data Quality Assessment (version QA00) of the Environmental Protection Agency (EPA). For AMPA we used 'trimmed mean', as 15% trimming may be a good estimator of the population mean in environmental data. Therefore, we trimmed 15% in the tails of our observations in the sensitivity analysis. In addition, we also performed a sensitivity analysis classifying the observations as detect/nondetect (i.e., binary). Both sensitivity analyses did not influence our results. Secondly, we performed a single imputation by replacement of fixed values (i.e., LLOQ/2) and found a significant association between AMPA exposure and telomere length. Thirdly, we applied a random imputation method using values between 0 and LLOQ (Bernhardt et al., 2015; HBM4EU, 2019; Pleil, 2016). This resulted in similar estimates and p-values. Lastly, we performed censored likelihood multiple imputation. The multiple imputation method has an advantage over the previous methods, as it provides a variance/confidence interval that can better understand the differences between different imputations (Den Hond et al., 2015). Trimming, classification, single, random, and multiple imputation performed similarly with respect to the significance of the models and the estimation in general, indicating that the association between AMPA exposure and telomere length is robust. For investigating the association of glyphosate and AMPA exposure, leukocyte telomere length was used. Although these telomeres are shorter compared with other tissues due to their high replication capacity, telomere length within individuals in different tissues were highly correlated (Friedrich et al., 2000; Martens and Nawrot, 2016). To correct for changes in the leukocyte cell proportions, we additionally adjusted our models for leukocyte count. Recently more cases of chronic kidney disease have been reported in agricultural communities. This has been linked with occupational exposure to glyphosate among Sri Lankan farmers (Gunarathna et al., 2018; Jayasumana et al., 2015). However, in our study, additionally adjusting for

cystatin C and alpha-1-microglobulin, indicators for renal function (Murty et al., 2013; Penders and Delanghe, 2004), did not affect our results.

CONCLUSION

Here, we found that urinary AMPA concentrations are associated with longer leukocyte telomere length in adults. To further understand the exact mechanism of glyphosate and AMPA toxicity on telomere length, more epidemiological as well as *in vitro* studies are required.

SUPPLEMENTAL INFORMATION

STUDY POPULATION

In total, 1369 subjects were invited by a letter from their general practice of whom 248 positively responded and 209 were included in the study, as described elsewhere (Vriens et al., 2019). Participation was solely based on the response to our letter; as such the participation rate was 18.1%. Proportional to the population number of each province, the number of subjects was geographically spread throughout Flanders. Field work was organised between May 2014 and November 2014.

MEASUREMENT OF MITOCHONDRIAL DNA CONTENT AND TELOMERE LENGTH

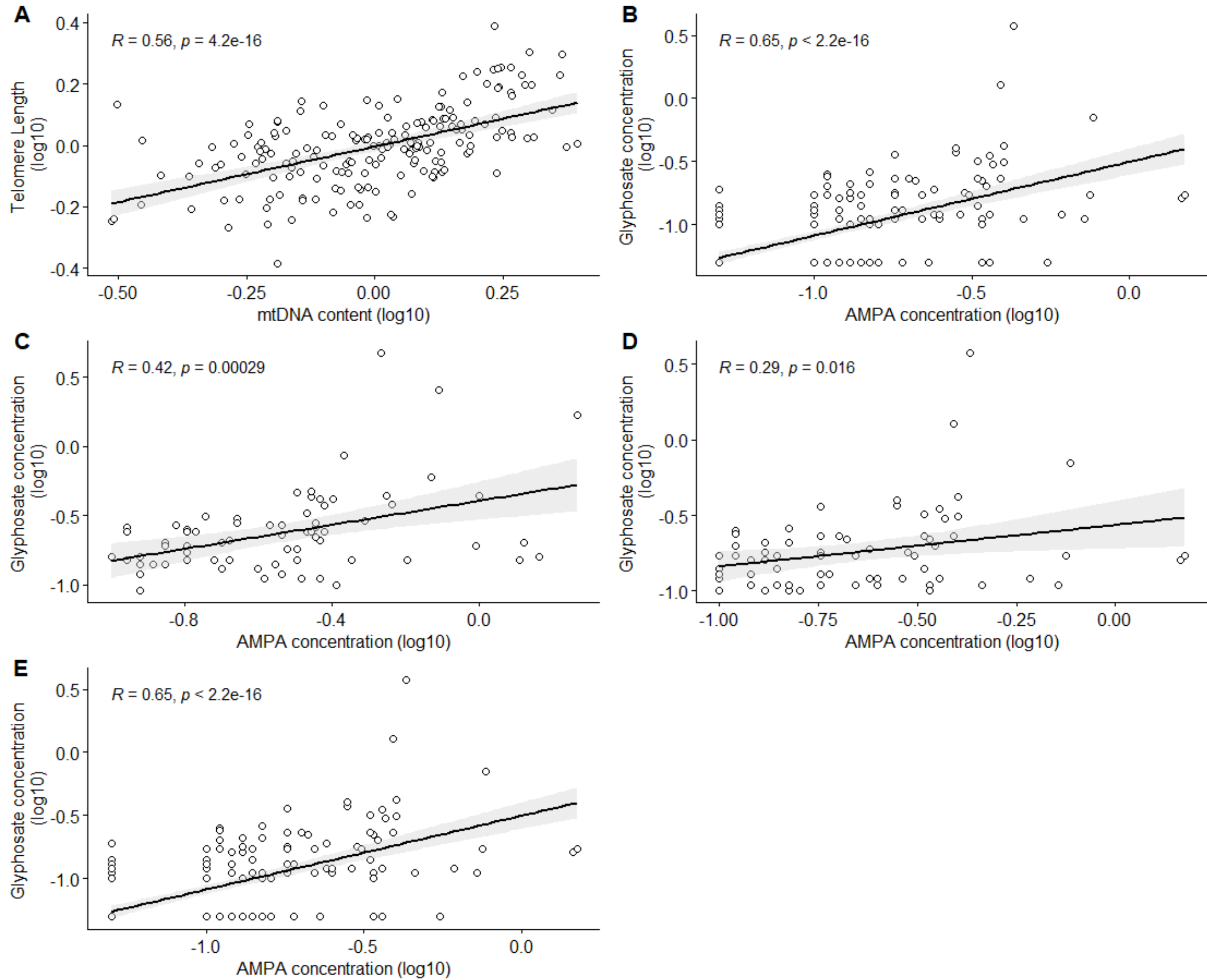
The measurement of mtDNA content and telomere length has been described elsewhere (Vriens et al., 2019). DNA was isolated from buffy coat, containing leukocytes, using the QIAamp DNA mini kit (Qiagen) according to the manufacturer’s instructions. The relative mitochondrial DNA (mtDNA) content and leukocyte telomere length were determined using quantitative PCR (qPCR) as previously described (Janssen et al., 2012; Martens et al., 2016). mtDNA content was measured by determining the ratio of two mitochondrial gene copy numbers (MTF3212/R3319 and MT-ND1) to one single copy nuclear control gene (RPLP0). Relative telomere length was determined by the ratio of the telomere sequence to the RPLP0 single copy nuclear control gene, proportional to the mean telomere length of the study population. qPCR reactions were carried out in a 10 µL volume on a 384-well plate using the 7900 HT fast real-time PCR system (Applied Biosystems). All reactions were carried out in triplicate, and every plate included three no-template controls. Each reaction contained 5 ng of DNA input and 1x QuantiTect SYBR Green Mastermix (Qiagen). Primer concentrations for the 36b4 assay were 300 nM of the forward and 500 nM of the reverse primers; for the mitochondrial assays, 300 nM for both forward and reverse primers; and 900 nM for the telC and 300 nM for the telG primers in the telomere assay. Primers for the mitochondrial genes (Janssen et al., 2012) and telomeres (Cawthon, 2009) are reported elsewhere. All thermal cycling profiles started with an initial step of 10 min at 95 °C for activation of the enzyme. For the 36b4 gene, this was followed

by 35 cycles of 15 s at 94 °C, 20 s at 62 °C, and 100 s at 74 °C; for the mitochondrial genes, this was followed by 40 cycles of 15 s at 95 °C and 70 s at 58 °C, and for the telomere this was followed by two cycles of 15 s at 94 °C and 2 min at 49 °C, then followed by 30 cycles of 15 s at 94 °C, 20 s at 62 °C, and 100 s at 74 °C. Raw qPCR data were evaluated using SDS software (version 2.3; Applied Biosystems). Only triplicates with a quantification cycle difference less than 0.5 were included in the study. qBASE software (Biogazelle) was employed to normalize the qPCR data.

SUPPLEMENTAL FIGURES AND TABLES

Supplementary Table 1: Study population characteristics of the trimmed population (n = 127).

Characteristic	Mean ± SD or *n* (%)
Age (years)	58.3 ± 4.1
Sex (female)	67 (52.8)
BMI	25.6 ± 4.5
Smoking status	
Never	51 (40.1)
Former smoker	58 (45.7)
Current smoker	18 (14.2)
Alcohol consumption	
Never	11 (8.7)
< Monthly	25 (19.6)
< Weekly	11 (8.7)
Weekly	80 (63.0)
Socioeconomic status	
Low	28 (22.1)
Middle	29 (22.8)
High	70 (55.1)
Season of sampling	
Winter	-
Spring	20 (15.8)
Summer	47 (37.0)
Autumn	60 (47.2)
Urine specific gravity	1.0 ± 0.01
Leukocyte count (amount/µL)	7000 ± 1550



Supplementary Figure 1: Pearson correlation plots of biomarkers of aging and markers of exposure. (A) Leukocyte telomere length and mtDNA content are positively correlated (R = 0.56; p < 0.0001). **(B)** Urinary glyphosate and AMPA concentration adjusted for urine specific gravity, including observations <LLOQ, are positively correlated (R = 0.55, p < 0.0001). The same positive correlation is shown for only observations >LLOQ adjusted for urine specific gravity **(C)**, for observations >LLOQ without adjustment for urine specific gravity **(D)**, and when using all observations without adjustment for urine specific gravity **(E)**.

Supplementary Table 2: The association between AMPA exposure and markers of biological aging in the trimmed population (n = 127), after single imputation (n = 181), and after random imputation (n = 181). Estimates were provided as the % difference (95% CI) of telomere length or mtDNA content from a doubling in AMPA concentration.

Models were adjusted for sex, age, BMI, smoking status, alcohol consumption, socioeconomic status, season, and urine specific gravity. mtDNA content has been additionally adjusted for platelet count.

	% difference (95% CI)	***p*-value**
Trimmed population		
Telomere length	8.67 (1.75 to 16.15)	0.01
mtDNA content	-0.07 (-10.37 to 11.34)	0.99
Single imputation		
Telomere length	2.95 (0.07 to 5.92)	0.045
mtDNA content	1.05 (-3.61 to 5.92)	0.66
Random imputation		
Telomere length	2.18 (0.04 to 5.70)	0.046
mtDNA content	0.63 (-4.07 to 4.97)	0.77

Supplementary Table 3: The association between glyphosate and AMPA exposure and telomere length using binary variables (n = 181). Estimates were provided as the % difference (95% CI) of telomere length or mtDNA content in AMPA concentrations >LLOQ compared to AMPA concentrations <LLOQ. Models were adjusted for sex, age, BMI, smoking status, alcohol consumption, socioeconomic status, season, and urine specific gravity.

	% difference (95% CI)	***p*-value**
Telomere length		
Glyphosate	5.20 (-3.84 to 15.08)	0.26
AMPA	10.66 (1.16 to 21.06)	0.03

ACKNOWLEDGMENTS

The FLEHS study was carried out by the Flemish Centre for Expertise on Environment and Health and was commissioned, financed and steered by the Ministry of the Flemish Community. Measurements of mitochondrial DNA content and telomere length were additionally supported by the EU Program “Ideas” (ERC-2012-StG 310898) and by the Flemish Scientific Fund (FWO G.0.873.11.N.10). Charlotte Cosemans is financially supported by the Centre for Environmental Sciences of Hasselt University. Bram G. Janssen is a postdoctoral fellow of the FWO (12W3218N). Dries S. Martens is a postdoctoral fellow of the FWO (12X9620N).

AUTHOR CONTRIBUTIONS

The FLEHS study was carried out by the Flemish Centre for Expertise on Environment and Health, in which TSN, WB, LB, EDH, VN, NVL, DC and GS have a coordinating role. TSN, MP, and CC designed the research hypothesis. DM and BGJ performed the mtDNA and telomere experiments. KS and EW critically revised the manuscript. CC analysed the data and interpreted the results. CC and MP drafted the article. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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