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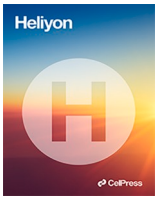

Research article

Short occupational exposure to glyphosate and its biomonitoring via urinary levels of glyphosate and metabolite AMPA (Amino-MethylPhosphonic acid), in Italian vineyard workers

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ABSTRACT

Glyphosate, an herbicide largely used in various contexts, can have adverse effects on human health. Although it is currently the most applied pesticide worldwide, few studies evaluated the extent of human exposure via biomonitoring. To expand such information, biological monitoring of exposure to glyphosate was conducted.

The study has a before-and-after design to demonstrate the immediate impact of short-term interventions. Accordingly, the urine concentrations of glyphosate and its main biodegradation product (amino-methylphosphonic acid- AMPA) were measured before and the day after the single herbicide application in 17 male winegrowers. Urine samples were analyzed by high performance liquid chromatography coupled with a triple quadrupole mass spectrometer equipped with an electrospray ionization source.

Glyphosate and AMPA were not detectable in pre-application urine samples (limit of quantification for glyphosate (LOQ_G) was 0.1 μg/L; limit of quantification for AMPA (LOQ_{AMPA}) was 0.5 μg/L). After application, glyphosate urinary levels were above LOQ_G in all workers. The median, min, and max values were 2.30, 0.51, and 47.2 μg/L, respectively. The same values were found for 50 %, 5 % and 95 % percentiles. After assigning numerical values, such as one half the LOQ, to each of the non-detects, the "z" of Wilcoxon matched-pairs signed-ranks test was −3.62 ($p = 0.0003$), suggesting the pre-application values being significantly lower than the post-application urinary glyphosate concentration. A similar analysis was not feasible with AMPA urinary levels, which were detectable only in 3 workers, after application. 12 (71 %) workers were significantly exposed to glyphosate, but adherence to the adoption of personal protective equipment was good: 14 (82 %) workers used gloves, 13 (76 %) used overalls and 13 (76 %) facial masks.

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Our data show that glyphosate can be absorbed by the workers after a single application and confirms the usefulness of biomonitoring in exposed workers. Further studies are needed in larger working populations and with multiple glyphosate applications, as well as to evaluate the correlations of glyphosate urine levels with exposure questionnaire data, in order to assess the actual relevance of risk and protection factors.

1. Introduction

Glyphosate is an herbicide used in various contexts such as agriculture, forestry, urban and home [1]. glyphosate is currently the most common pesticide worldwide and its extensive use is due to several characteristics, such as broad spectrum, high efficacy, and low cost.

[2].

The occupational exposure to glyphosate has been correlated with various health effects. Its role as carcinogen is still unclear. Some studies have found a link between glyphosate and non-Hodgkin lymphoma [3,4], in contrast with results reported by other authors [5,6]. On one hand, glyphosate has been classified as a probable human carcinogen by the International Agency for Research on Cancer (IARC) in 2016 [1,7]. On the other hand, other European and International Agencies classified glyphosate as non-carcinogenic [8]. Furthermore, some studies suggest a positive association with Alzheimer's disease, autism, and Parkinson's disease [9–11], asthma, rhinitis [12–14], hypothyroidism and infertility [15,16].

This herbicide is currently permitted in the EU, where the global glyphosate market increased from almost 24 billion dollars in 2016 to 34 billion in 2022, with a growth rate of 6 % per year [17]. In the Veneto region, 446 tons of glyphosate were consumed in 2015, mainly in vineyards [18].Currently, some countries in EU have provided precautionary measures. In Italy, the herbicide's application is allowed with restrictions in agriculture, while it is prohibited in the green areas of the urban context [19]. Occupational exposure to glyphosate occurs mainly via inhalation, dermal contact, ocular contact during manufacture, transport, mixing, loading, application, and disposal processes. In addition, glyphosate can be ingested as a result of accidental oral exposure or through the diet [20].

Only a small fraction of glyphosate is metabolized by the human body, so most remains detectable in the urine [21]. Its excretion has two phases, a shorter one with a half-life around 6 h and a longer one with a half-life around 18–33 h [22–24]. The glyphosate's major biodegradation product in mammals is amino-methyl-phosphonic acid (AMPA) that is also excreted with urine [25,26].

Due to the short biological half-life of glyphosate, biomonitoring should be performed immediately after the exposure. Biomonitoring, namely measuring the levels of chemicals in biological matrices such as urine or blood, is the gold standard to assess the occupational exposure of glyphosate. The literature highlights several limitations to be considered while interpreting the biomonitoring studies, such as: small sample size, low representativeness, different case studies, different ways to characterize exposure, selection bias, as well as analytical methods with different sensitivities [25].

Biomonitoring is not generally feasible for retrospective studies. Several studies assessed the exposure in occupational settings through questionnaires [5,6,27–29]. Dosemeci et al. (2002) proposed an algorithm to estimate lifetime average exposure intensity to pesticides, using questionnaire information [29]. Acquavella et al. (2004) applied this method also for glyphosate exposure, showing a moderate correlation between algorithm scores and glyphosate urine concentrations [30]. The authors concluded that collecting data on pesticides exposure using questionnaires is useful in epidemiological studies, but the assessment could be affected by appreciable exposure misclassification.

Regarding glyphosate exposure, in Italy vineyard workers (VW) are one of the most exposed professional categories. An Italian study reported that glyphosate was used by more than 80 percent of vineyard workers living in a valley in Northern Italy and glyphosate levels above the detection limit were found in nearly half of the vineyard groundwater samples analyzed [31]. However, to the best of our knowledge, no studies are available on biomonitoring of glyphosate in VW.

The present paper aims to assess and evaluate occupational exposure and perform the biomonitoring of glyphosate, describing urine concentrations of the pesticide and its main biodegradation product AMPA in a sample of VWs before and after a single treatment of vineyards. Other outcomes, including immunological function (IL- 4, IL-5, IL-8, IL-12, IL-17, IL-33, IFN-γ and Th cells subpopulations), detection of transcriptional and post-transcriptional alterations (miRNA), and genotoxic effects by Comet-assay on lymphocytes DNA were assessed before and after glyphosate exposure. The corresponding results will be reported in a companion paper.

2. Material and methods

2.1. Study design

This study has a before-and-after design, that is most useful in demonstrating the immediate impacts of short-term interventions.

2.2. Subjects

At enrollment phase, a series of meetings were scheduled, involving the Local Health Authority as well as bilateral boards of Trade

Unions and farm owners, to raise the awareness on glyphosate risks, to provide practical guidance for the glyphosate use, and to explain the aims of the study. The boards were asked to contact VW and elicit their interest in participating. Subjects who accepted to participate in the study were invited to the Section of Occupational Medicine of the University of Verona. They were asked to provide the pesticide official health authority registers, containing data about applications during the previous year, information regarding the workplace (location of the farm, owner's data, the holder of the pesticide license, farm extension, type of sprayer machines used).

The following exclusion criteria were applied.

- having used glyphosate recently for any reason;
- presence of chronic degenerative diseases;
- current treatment with chemotherapeutic drugs and/or nitro heterocyclic compounds;
- occupational exposure to chemical agents in the previous six months.

Twenty-six male volunteers, including 25 vineyard workers and 1 fruit grower, belonging to 25 farms located in the Verona area, North-Eastern Italy, were enrolled. After a general clinical examination, participants were asked to collect a 24 h urine sample and fill in a questionnaire, before and immediately after the use of glyphosate. Among 26 participants, two were not able to deliver their post-exposure urines. Twenty-four subjects delivered the urine containers to the laboratory but, due to some technical issues, analyses were feasible only on 17 samples. All samples were collected from February to July 2018. Therefore, the statistical analysis covered information collected on questionnaire (personal and health characteristics, characteristics of farm, farmers, and work practices) as well as urinary concentrations of glyphosate and AMPA before and after its application for 17 farmers.

2.3. Questionnaires

Applicators followed their usual procedures. Information on the different phases of application process was obtained using interviewer-administered questionnaires. Participants filled in two questionnaires designed ad hoc, one before and one after the use of glyphosate. The survey was devised based on ad-hoc questionnaires, specifically adapted for this research and previously pilot tested on agricultural workers, in order to assess the respondents' compliance and the mean time to fill in (about 20 min).

The first questionnaire included 40 items, divided in three sections (more details are available upon request to the Authors).

- personal data and main characteristics of the farm;
- clinical history including occurrence of chronic diseases and personal habits (e.g., alcohol and cigarette consumption);
- intensity-related glyphosate exposure questions. In agreement with the quantitative approach of Dosemeci et al. (2002) [29] ([MIX] represents exposure from mixing and loading operations prior to application, [APPLY] represents exposure from applying pesticides, [REPAIR] represents exposure from contact with contaminated surfaces during the maintenance of pesticide application equipment, [HYG] investigating washing clothes after use of pesticides, [PPE] use of personal protective equipment represents an exposure reduction factor).

The second questionnaire was filled after glyphosate treatment and consisted of 15 questions investigating technical information on glyphosate, duration and extent of the application, any accidental spills, or direct contacts with glyphosate.

2.4. Physical examination

A medical examination was carried out by physicians, including full occupational and clinical history.

2.5. Urine collection

Participants were informed by medical staff and thorough information sheets on how to collect the urine with particular attention to avoid contamination. In this way, two 24-h urine collections were performed.

1. After excluding the first morning urination, the first one was performed between the medical examination and the first application.
2. The second collection was carried out on the day of glyphosate application, starting in the morning, and ending the next day including the first urination of the day.

The containers were stored at +4 °C during the collection and were delivered to the laboratory within 6 h after the end of the collection. Then laboratory staff aliquoted each urine sample in three 100 ml tubes and stored them at −80 °C.

2.6. Analytical methods

The analyses were performed at the laboratory of environmental and occupational toxicology, Department of Clinical and Community Sciences, University of Milan.

Briefly, the frozen urine sample was towed at room temperature. Urine was added with a mixture of isotopically labelled internal standards (Glyphosate-2-13C,15N from Sigma Aldrich and AMPA-13C,15N,D2 from Cerilliant) and was purified using solid phase

extraction with the SampliQ Si-SAX cartridge (500 mg * 3 mL, Agilent Technologies, Cernusco sul Naviglio, Italy). The extract was analyzed by high performance liquid chromatography (Agilent Technologies 1260, Cernusco sul Naviglio, Italy) equipped with a Raptor Polar X column (50 × 2.1 mm, 2.7 μm particle size, Restek, Cernusco sul Naviglio, Italy) using a mixture of 0.5 % formic acid in water and CH3CN as eluent. The detector was a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500; Sciex, Monza, Italy) equipped with an electrospray ionization source (ESI) operating in the negative ionization mode. The principal ionization source parameters were: gas 1 pressure 80 psi, gas 2 pressure 60 psi, curtain gas pressure 20 psi, heater temperature 600 °C, and entrance potential 10 V. The two most intense multiple reaction monitoring transitions for each native analyte were recorded and used for quantitation and qualification. The method was validated according to US Food and Drug Administration FDA [32]. The limit of quantification for glyphosate (LOQ_G) was 0.1 μg/L; the limit of quantification for AMPA (LOQ_{AMPA}) was 0.5 μg/L. LOQ was determined using the equation: LOQ = (5SEq + q)/m, where SEq is the standard error of the intercept q, and m is the slope of the linear regression. At the LOQ, the precision was 2.3 % for glyphosate and 3.8 % for AMPA, while the accuracy was 112 % for glyphosate and 101 % for AMPA [33].

2.7. Statistical analysis

A measurement of urinary glyphosate and AMPA was taken on each subject before and after the herbicide treatment. Several values of glyphosate and AMPA were not detected. It is technically impossible for a laboratory analysis to confirm the complete absence of a chemical or compound of interest. Since the true level is unknown, laboratories report the nonzero value as below LOQ (limit of quantification), representing the lowest concentration which can be detected and quantified with a specified degree of precision, typically 20 %. The analytical method used in our study let us to achieve a $LOQ_G = 0.1$ μg/L and $LOQ_{AMPA} = 0.5$ μg/L. Using a simple substitution method, we assigning surrogate numerical values (such as one half the LOQ) to each of the non-detects [34].

Due to non-normal distribution of variables, to examine the equality of matched pairs of observations we used the Wilcoxon matched-pairs signed-ranks test. The null hypothesis was that the median of the "after-before" differences is zero; no further assumptions are made about the distributions.

Furthermore, the STATA command "kdensity, normal" was used to produce kernel density estimates and graph the result; "normal" requests that a normal density be overlaid on the density estimate for comparison. We also used the commands: "ladder" to find a subset of the ladder of powers that converts a given variable into a normally distributed variable; and "summarize, detail" to calculate a variety of univariate summary statistics including skewness and kurtosis.

Table 1
Main characteristics of farms and farmers with biomonitoring data (17 subjects): number (percentage); mean and standard deviation (SD).

FACTORS	N (%)	Mean ± SD
Personal habits and general health characteristics		
Age (years)		49.5 ± 11.1
Weight (cm)		178.2 ± 5.9
Height (kg)		84.1 ± 12.5
BMI		26.5 ± 3.6
Married	15 (88 %)	
Current smokers	8 (47 %)	
Pack-years		8.4 ± 7.6
Alcohol drinkers	16 (94 %)	
Wine (glasses/day)		2.1 ± 0.7
Coffee drinkers	13 (76 %)	
Coffee (cups/day)		2.3 ± 0.7
No chronic disease	10 (58 %)	
No skin disorders	17 (100 %)	
Characteristics of farm, farmers, and work practices		
Farm hectares		18.1 ± 12.6
Farm employees		1.8 ± 1.1
Employment (farm owner versus others)	15 (88 %)	
Training in Agriculture	17 (100 %)	
Licensed	17 (100 %)	
MIX = High exposure	2 (12 %)	
APPLY = High exposure	12 (71 %)	
REPAIR = High exposure	10 (59 %)	
HYG = High exposure	12 (71 %)	
OVERALLS = High protection	13 (76 %)	
GLOVES = High protection	14 (82 %)	
BOOTS = High protection	2 (12 %)	
MASK = High protection	13 (76 %)	
Spill occurrence	0 (0 %)	
Hectares sprayed		7.6 ± 4.3
Hours of glyphosate treatment		6.6 ± 1.9
Volume of glyphosate spread (L)		1240 ± 907.8

3. Results

Table 1displays personal data and working practices of 17 farmers with biomonitoring data. All subjects were males, mostly middle-aged, married, with BMI <30, with moderate consumption of tobacco, alcohol and coffee, and few chronic health problems. The latter were: hypertension, allergic asthma (1 case), hypercholesterolemia, allergy to grass pollen and gastro-esophageal reflux. No skin disease neither use of skin drug was reported. Size of farm was above than the average in Italy and the applicator was mostly the farm owner. All applicators of glyphosate were certified as pesticide applicator and most of them reported training in agriculture. glyphosate was spread in early springtime and in hilly terrains. Based on literature data, APPLY (in particular: tractor with open or non-existent cabin; and manual delivery with a traditional shoulder model with pumping lever) along with REPAIR (cleaning of internal tank; and cleaning nozzles) could be the work phases involving the highest exposure. On the other hand, the workers wore PPE to protect airways and skin (the main absorption routes) during herbicide spreading. The answers to the questionnaires revealed a very good adherence to the PPEs including suits, gloves, and facial mask among our VWs. In fact, 74 % of workers used at least two types of PPE and only a 26 % of them used only one type. Correlations of glyphosate and AMPA urine levels with questionnaires data could demonstrate the actual relevance of the different risk and protection factors; however, this evidence will be reported in a next companion paper.

3.1. Glyphosate and AMPA urine levels

Individual glyphosate and AMPA urine levels are reported in Table 2. Before application, glyphosate and AMPA were not detectable in any of the 24 h urine aliquots. In the samples collected after application, glyphosate levels were above the LOQ for all the subjects, while AMPA was detected in three subjects (number 12, 15, and 17) with glyphosate levels above 5 μg/L.

The last line of Table 2 reports for glyphosate concentration in post-shift urines the indices of central tendency (median) and statistical dispersion (min, max) in the whole series of 17 farmers. The median was 2.30 μg/L, the min value 0.51 μg/L, and max value 47.2 μg/L. Similar values were found for 50 %, 5 % and 95 % percentiles.

After assigning numerical values, such as one half the LOQ, to each of the non-detects, the "z" of Wilcoxon matched-pairs signed-ranks test was −3.62 ($p = 0.0003$). The minus sign of z suggested the pre-exposure values being significantly lower than the post-exposure urinary glyphosate concentrations.

A comparable statistical analysis was judged not feasible for AMPA urine concentrations. Any statistical test could not provide valid results because AMPA values were detected only in three out of 34 (17 before and 17 after application) urine samples.

The major outcome of the present study was glyphosate concentration in post-exposure urines. The distribution of the original variable was asymmetrical and right skewed (Fig. 1, left panel). With natural logarithm transformation of data, distribution turned to normal (Fig. 1, right panel). The chi-square test for departure from normal distribution was 26.14 ($p < 0.0001$) for the original variable and 2.16 ($p = 0.340$) for log-transformed variable. Pearson's median skewness was 3.3265, higher than the cutoff of 0.4, with a positive sign evidencing a positive asymmetry with a long tail on the right side of distribution.

The most common measures of central tendency and dispersion for the distribution of 17 post-exposure urine concentrations of glyphosate were: arithmetic mean (M) and standard deviation (SD); geometric mean (GM) and geometric standard deviation (GSD); percentile 25 %, 50 % (median) and 75 % percentile. The GM (=2.44 μg/L) was comparable to the median (=2.30 μg/L); both values

Table 2
Individual glyphosate and AMPA urine levels collected before and after a single application of glyphosate; position and dispersion indices for Glyphosate post-shift urine concentration in the whole sample.

Subjects	Glyphosate (μg/L)		AMPA (μg/L)	
	Before	After	Before	After
1	<LOQ	4.00	<LOQ	<LOQ
2	<LOQ	9.49	<LOQ	<LOQ
3	<LOQ	2.65	<LOQ	<LOQ
4	<LOQ	0.54	<LOQ	<LOQ
5	<LOQ	2.00	<LOQ	<LOQ
6	<LOQ	5.92	<LOQ	<LOQ
7	<LOQ	2.81	<LOQ	<LOQ
8	<LOQ	2.30	<LOQ	<LOQ
9	<LOQ	1.40	<LOQ	<LOQ
10	<LOQ	0.59	<LOQ	<LOQ
11	<LOQ	0.53	<LOQ	<LOQ
12	<LOQ	47.16	<LOQ	2.05
13	<LOQ	0.89	<LOQ	<LOQ
14	<LOQ	1.89	<LOQ	<LOQ
15	<LOQ	10.18	<LOQ	1.35
16	<LOQ	0.51	<LOQ	<LOQ
17	<LOQ	5.03	<LOQ	1.15
All	–	median, min, max 2.30, 0.51, 47.2	–	–

Note: The limit of quantification for glyphosate (LOQ_G) was 0.1 μg/L; the limit of quantification for AMPA (LOQ_{AMPA}) was 0.5 μg/L.

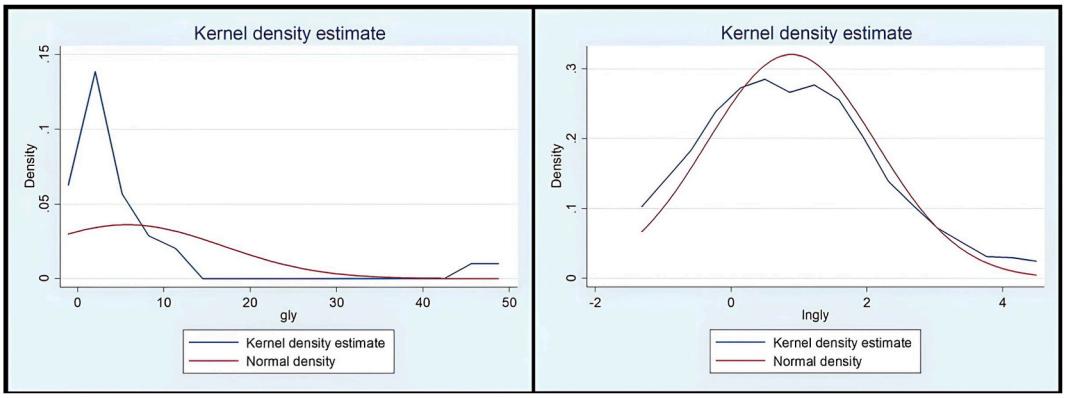

Fig. 1. Distribution of glyphosate post-exposure concentration: original (left panel) and log-transformed values (right panel).

were much lower than arithmetic mean (=5.76). The interquartile range (IQR) is a measure of statistical dispersion; it is defined as difference between the 75th and 25th percentiles of the data, so IQR = (5.03–0.89) = 4.14 μg/L. The latter value was closer to the GSD (=3.47) than SD (=11.07). Based on these measures of dispersion, the central tendency was strong for log-transformed but weak for the original data. Lastly, the ratio GM/LOQ_G was 24.4; the former was about 25 times higher than the latter.

4. Discussion

The results of this analysis provide an estimate of the amount of glyphosate that was absorbed by the workers after a single application, by a population of 17 male VWs from the province of Verona (North-Eastern Italy).

Although it has been released since the 1970s, becoming the most widely used herbicide in the world [35], biomonitoring data on workers exposed to glyphosate – and especially in VW - are still insufficient and scanty [25].

Our research also aimed at discussing the overall value of glyphosate biomonitoring data in the field of occupational health. Accordingly, we summarized in Table 3 (inspired by Connoly et al., 2020 [25], modified and updated) previous occupational biomonitoring studies that examined urinary glyphosate and, in some cases, its metabolite AMPA [21–24,30,36–41].

Some researchers carried out biomonitoring in agricultural workers other than vineyard, detecting an increase in urine glyphosate levels after pesticide application. Acquavella et al. (2004) found detectable levels of glyphosate in the urine in the 60 % of enrolled farmers. The prevalence of urine samples with detectable glyphosate levels showed a large variability between countries (87 % in South Carolina vs 36 % in Minnesota, USA) [30]. Connolly et al. (2017) analyzed urine sample before and after glyphosate application in horticulturalists. glyphosate was detectable in 35 % urine samples before glyphosate application, raising to 55 % after. The higher percentage of pre-application samples in which glyphosate was detectable is likely related to the exclusion criteria applied in that population [38]. Indeed, Connolly et al. (2017) [38] also included workers who had used glyphosate on previous days. This hypothesis seemed to be confirmed in the following paper by Connolly et al. (2018) [37], in which the Authors reported that pre-exposure values were higher in those who had performed job tasks involving the use of glyphosate in the previous days. In the study of Perry et al. (2019), only post application samples were available founding detectable glyphosate values in the 39 % of sample collected 8 h after the first seasonal glyphosate use. The only subject who also tested positive for AMPA was the one with the highest glyphosate value in urine, suggesting a possible correlation between higher levels of exposure and the presence of AMPA [41]. Furthermore, Zhang et al. (2020), in a study involving workers assigned to glyphosate production, found a significant correlation between glyphosate and AMPA concentration in urine samples, and both were related to exposure intensity. In our study, we could detect glyphosate in all post treatment urine samples; this is due to the lower LOQ in comparison with studies conducted in the past, while no glyphosate was detected in pre-exposure sample, confirming that the application of glyphosate was not performed by these workers in the days before the study. In agreement with previous results, subjects with the highest level of glyphosate were those for which also AMPA was detectable [42].

On the other hand, Jauhiainen et al. (1991) and Lavy et al. (1992) did not find detectable levels in forest workers to glyphosate during brush saw spraying task and in conifer seedling nursery worker, respectively [39,40]. However, these results, elaborated in the '90, may have been affected by the higher LOD, due to technical limitations. Anyway, the literature highlights that the prevalence of detectable glyphosate and AMPA levels varies significantly among different job tasks, proving the need of data on each specific work setting [25].

4.1. Strengths and limitations

As for the strengths, to the best of our knowledge, the current research would be the first report concerning the detection of urinary concentration of both glyphosate and AMPA among Italian vineyard workers. Moreover, the analytical method used in our study let us to achieve a lower LOQ, improving the sensitivity of the analysis; LOQ_G = 0.1 μg/L and LOQ_{AMPA} = 0.50 μg/L.

Table 3
Main features of the studies regarding occupational exposure to glyphosate.

Authors	Population	Country	Urine collection	Detection Method	Glyphosate Concentration (μg/L)			AMPA Concentration (μg/L)		
					LOD/ LOQ	% above LOQ/LOD	Results	LOD/ LOQ	% above LOQ/ LOD	Results
Jauhaianien, A. et al., 1991	5 Forest workers +5 controls	Finland	Workday end and after a 3 week period	GC with a 63Ni-electron capture detector	LOD 1	0	/	LOD 0.5	0	/
Lavy, T.L. et al., 1992	14 Conifer Seedling Nursery Workers	United States	24 h (each day for a minimum of 8 weeks)	NR	LOQ 10	0	/	NS	NS	NS
Acquavella, J.F. et al., 2004	48 farmers, 48 wifes and 79 children	United States	24 h (the day before spraying, the day of sprying and te 3 following days after)	HPLC following ion exchange	LOD 1	Farmer 60 % Wife 4 % Child 12 %	GM (farmers) 3.2	NS	NS	NS
Curwin, B. et al., 2007	25 farm households +25 non farm households	United States	Two spot urine samples (one in the evening and one the following morning)	Immunoassay	LOD 0.9	~77 %	**GM (farm)** Mother 1.5 Child 2.0	NS	NS	NS
Mesnage, R. et al., 2012	Farmer's Family (1 farmer, 1 wife and 3 children)	France	24 h (the day before and 2 days after spraying)	HPLC-ESI-MS	LOD 1 LOQ 2	NR	**M (Farmer)** Day before application 4.35 1 day after 0.95 2 days after 1.90 **M (Child)** 2 days after 2.00	NR	0	/
Jayasumana, C. et al., 2015	10 healthy farmers from an area with chronic endemic kidney disease	Sri Lanka	Non-fasting spot urine samples collected in the morning	ELISA	0.6	NR	MED 82.6 (17.1–195.1)	NS	NS	NS
Connolly, A. et al., 2017	17 workers of horticulture amenity gardening	Ireland	Spot urine: before and after the work tasks	LC-MS/MS	LOQ O.5	55 %	GM 0.66 M 1.35	NS	NS	NS
Connolly, A. et al., 2018	20 workers of horticulture amenity gardening	Ireland	Individual full urinary void spot samples (before and after the work tasks and the following morning)	LC-MS/MS	LOQ O.5	93 %	GM 1.90 M 2.53	NS	NS	NS
Perry, M.J. et al., 2019	18 farmers +17 non-applicators	United States	Spot urine, 8 h post application	LC-MS/MS	LOD 0.4	39 %	M 4.04	LOD 1	6 %	M 4.1
Zhang, F. et al., 2020	134 workers during glyphosate production	China	Samples were collected within 1 h of the end of shifts	GC-MS	LOD 20	~87 %	GM 262	LOD 10	~81 %	GM 72
Balderrama-Carmona, A.P. eta al., 2020	30 agricultural workers	Messico	24 h urine	HPLC	LOD 5	0	/	LOD 15	6 %	NR
Sasivimol Bootsikeaw et al., 2021	43 vegetable farmers	Thailand	3 Spot urine samples (the first morning before glyphosate spraying day, the end of glyphosate spraying event and the next morning after glyphosate spraying day)	HPLC–FLD system	LOD 1	NR	GM (μg/g creatinine) Before glyphosate spraying day 28.21 At the end of glyphosate spraying event 38.66 Next morning after glyphosate spraying day 37.27	NS	NS	NS

(*continued on next page*)

Table 3 (*continued*)

Authors	Population	Country	Urine collection	Detection Method	Glyphosate Concentration (μg/L)			AMPA Concentration (μg/L)		
					LOD/ LOQ	% above LOQ/LOD	Results	LOD/ LOQ	% above LOQ/ LOD	Results
Connolly A et al., 2022	54 non farm families and 14 farm families (180 non farm subjects-46 farms)	Ireland	glyphosate spraying day, the end of glyphosate spraying event, and at first morning voids in	GC-MS/MS	LOD 0.05	20 % non farm, 43 % farm;	no significant differences between farm and non-farm families	LOD 0.05	59 % non farm, 57 % farm	no significant differences between farm and non-farm families
Present contribution	17 winegrowes	Italy	the next morning after glyphosate spraying day.	LC-MS/MS	LOQ 0.1	100 %	**After Application** GM 2.44 M 5.76 MED 0.50	LOQ 0.5	~18 %	**After Application** GM 0.60 M 0.68 MED 0.50
Abbreviations:	NR: Not Reported NS: Not Studied MED: Median M: Mean GM: Geometric Mean		HPLC: High-Performance Liquid Chromatography HPLC-ESI-MS: Liquid chromatography, linear ion trap Mass Spectrometry LC-MS/MS: Liquid Chromatography tandem Mass Spectrometry GC-MS: Gas Chromatography-Mass Spectrometry							

Despite the unavailability of a few urinary samples, our sample size was like those found in previously published studies [21,24,37–41,43]. In such studies, the population size varied between 5 and 20 subjects and, only in three studies concerning applicators, the sample size was higher [22,30,36]. Another report describing the urinary concentrations of 134 workers involved in the industrial process of pesticide production, the population is also larger than ours [41].

Another strength is the investigation of AMPA urinary levels. In fact, only 6 out of 12 other studies have measured this metabolite and only 5 were able to finding it [21,22,24,39,41,42]. Our results showed that 18 % of urine samples were above the LOQ for AMPA after glyphosate application. Slightly lower values were described in Balderrama-Carmona et al. (2020) [22] and Perry et al. (2019) [41], who respectively detected levels above LOD respectively in 7 % and 6 % for AMPA.

On the contrary, Juahiainen et al. (1991) [39] and Mesnage et al. (2012) [24] did not detect AMPA in urine samples. It is here relevant to point out that, in the last two researches, the LOD was 50 μg/L in the former study and not specified in the latter.

Zhang et al. (2019) [42] reported, in a population involved in pesticide production, the highest positivity rate above LOD (81 %). However, this Chinese study is difficult to compare to the others, because it analyses occupational exposure resulting from glyphosate industrial production and not from its use and spreading in agriculture.

In a recent survey, Connolly et al. (2022) [21], evaluated glyphosate and AMPA concentration among 54 non-farming and 14 farming families. Among farmers, the urine concentration of glyphosate was above the LOQ in 43 % of the subjects, whereas was 57 % for AMPA. This is peculiar and in contrast with results of our and previous studies; maybe this is due to the very high sensitivity of the method used by Connolly et al., with LOQ of 0.05 μg/L for both glyphosate and AMPA; another possibility is the presence of other sources of exposure to AMPA, as it may be also present in the environment following the use of detergents.

As for the limitations, a few considerations should be mentioned from the statistical point of view. Since the index intervention was an exposure (not a treatment) the relevant difference was “after before” rather than “before – after”. Our data set included very low concentrations of glyphosate (measure unit = μg/L) and the whole series of before-values were non-detects (Table 2). Since the paired *t*-test is based on means and variances, the simple substitution of non-detects with a fixed numerical value could produce erroneous results [44]. Additionally, subjects were not a random sample of the population; the difference of matched samples was not normally distributed and presented an extreme outlier (data not shown). In view of all the above, the *t*-test for paired samples could not be valid and was rejected. By contrast, no assumptions about the distributions are needed for the Wilcoxon signed-ranks test. The statistics “z” of Wilcoxon signed-ranks test was highly significant (two-tail p-value = 0.0003), suggesting the post-exposure urinary glyphosate concentration being significantly higher than pre-exposure values.

In our study, farmers had been given a test and repeated the same test following the day of occupational exposure to glyphosate. In this situation, the evidence would be strong that occupational exposure caused the increase of the urine test score. Little else could have caused the observed change over the course of one day.

It should be underlined that the VW applied glyphosate only once. This element cannot be compared with other studies, because the frequency of application was not reported. Moreover, the urine samples were collected only once, so we could not investigate the trend of glyphosate and AMPA over time.

Also, despite several attempts via the local health authority as well as workers and farmers associations, it was not available the number of workers actually in charge of glyphosate application, therefore leaving uncertainty regarding the representativeness of the study sample. However, informal estimates showed that approximately 200 workers were involved in such activity in the area, therefore our population can be considered representative.

It should also be noted that, in the general population, the main sources of exposure to pesticides are contaminated food and water [45,46]. This variable was not investigated in our cohort leading to a possible overestimation of occupational exposure. Furthermore, all VW were male, and no gender variability could be appraised [45], in fact, reported a positive association with urinary creatinine concentration, reflecting a sex-related difference in urinary glyphosate and AMPA.

It should also be commented that the glyphosate toxicokinetic has not yet been fully clarified. On this topic, Zoller et al. (2020) [26], and Connolly et al. (2019a, 2019b) [47,48] estimated a half-life between 5.5 and 10 h. On the contrary, Faniband et al. (2021) [49] and William et al. (2000) [50], found a half-life characterized by two phases: a fast one, between 6 and 9 h, and a slow one, that can exceed 24 h. In addition, Zoller et al. (2020) [26] and Faniband et al. (2021) [49] studies agree that 1–6% of an incorporated glyphosate dose is excreted as glyphosate in urine. They also agree that the conversion of glyphosate to AMPA in humans is marginal. As a result, it is difficult to compare data from occupational studies which are often conducted with different sampling strategies and/or different analytical methods. Moreover, we could assume that, in our study, a mixture of all three potential exposure pathways might have occurred (i.e., via inhalation, dermal and oral routes), that are difficult to disentangle.

It is also conceivable that the use of different types of PPEs, different ways of pesticide dispersion and different tasks in the work chain, which may include not only dispersion, but also mixing and dilution, may cause different levels of exposure. Information about PPE used during the glyphosate spreading, application techniques, hygiene of dispersion tools, and accidents, were collected with questionnaires, however, all these data, together with their correlation with glyphosate and AMPA urine levels, will be reported in a companion paper.

5. Conclusions

Our study is the first to report assessment of glyphosate exposure following brief application of glyphosate in vineyard workers, measured via biomonitoring of glyphosate and its metabolite AMPA and showing some absorption of the xenobiotic. The research confirms the usefulness of biomonitoring in exposed workers. Further studies are needed in larger working populations, to evaluate the exposure after multiple applications, as well as the correlations of glyphosate urine levels with exposure questionnaire data, in order to

assess the actual relevance of risk and protection factors.

Ethics

This study was reviewed and approved by Comitato Etico per la Sperimentazione Clinica delle province di Verona e Rovigo, with the approval number: N. 32506, dated July 14, 2015.

All participants provided written informed consent to participate in the study and for their data to be published.

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Data availability statement

Data associated with our study has not been deposited into a publicly available repository but they will be made available upon request to the Authors.

CRediT authorship contribution statement

Stefano Porru: Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **Melissa Ferrian:** Writing – original draft, Validation, Investigation, Formal analysis. **Giuseppe Mastrangelo:** Writing – original draft, Formal analysis, Data curation. **Diego Capovilla:** Writing – original draft, Formal analysis. **Emanuela Corsini:** Writing – review & editing, Validation, Conceptualization. **Silvia Fustinoni:** Writing – review & editing, Validation, Investigation. **Manuela Peruzzi:** Writing – review & editing, Resources. **Claudio Colosio:** Writing – review & editing, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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