HAIR AS A MATRIX TO EVALUATE CUMULATIVE AND AGGREGATE EXPOSURE TO PESTICIDES IN WINEGROWERS

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ABSTRACT

Introduction. Vineyard is a crop where a large number of pesticides are applied; exposure to pesticides may occur in farmers and the general population living close to the treated area. This work aimed to investigate hair as a matrix for the assessment of cumulative and aggregate exposure to pesticides in potentially exposed individuals.

Methods. Twenty agricultural workers (AW), 4 agricultural worker relatives (AR), and 5 research staff members (RS) were involved in the study. Hair samples were collected before and after the application season (PRE- and POST-EXP samples) to obtain 18 paired samples. Records with the name and the quantity of applied pesticides were obtained; twenty-seven pesticides were measured in hair by solvent extraction and LC-MS/MS.

Results. During the study season, AW applied 14 different pesticides with median amount ranging from 12 to 7200 g. The most popular pesticides were dimethomorph, penconazole, cyazofamid, fenamidone and quinoxyfen, applied from 94 to 69% of AW. In AW, in PRE-EXP samples the majority of used pesticides was detectable (with detection rates from 6 to 88%), with median concentrations of few pg/mg hair; in the POST-EXP samples the frequency of detected values increased (from 25 to 100%), with median concentrations up to two orders of magnitude higher. In AR, most pesticides were quantifiable only in POST-EXP samples and with lower concentration in comparison with AW; in RS, in both PRE- and POST-EXP samples only a few pesticides were quantifiable with very low levels. In AW, a linear correlation ($r = 0.682$ on log-transformed data, $p < 0.01$) was found between the total amounts of applied pesticides during the season and their concentration in hair.

Conclusion. The study shows that the majority of assessed pesticides was incorporated into hair of AW and AR. The increased frequency of detection and level at the end of the season and the correlation between pesticide in hair and the amount of applied pesticides, reinforce the use of hair for quantitative biomonitoring of cumulative exposure to pesticides.

1. INTRODUCTION

Vineyard is a cultivar in which several pesticides are used in different growing phases: herbicides are applied early in the season to keep the vineyard ground clean from weeds; fungicides are applied during the growing phase to fight molds which infest leaves and inflorescences during the wet season, and finally insecticides are used to avoid the attack of insects to the grapes in the last part of the growing period. Vineyard is a relevant crop in Italy, and in Lombardy, Northern Italy, there are 23.182 hectares cultivated with vineyards, which represent about 2.4 % of used agricultural surface (ISTAT, 2010).

Due to the biological activity of pesticides against several living organisms, and their intentional spread in environment, there is a large concern for human exposure and their consequent toxic actions.

In agricultural workers the professional use of pesticide causes the major source of exposure. Exposure mainly occurs via dermal penetration, while a minor amount of pesticides enters the body via inhalation (Baldi et al., 2006). The duties that potentially cause major exposures are mixing and loading, application, maintenance of the equipment, and re-entry activities (Baldi et al., 2014). In the general population residing near the treated crops, exposure may occur via inhalation of pesticide during the application, or via skin absorption, following the contact with contaminated surfaces. For both the agricultural workers and the general population diet may represent an additional exposure source (van Klaveren et al., 2015).

Exposure assessment is a relevant step toward risk evaluation. Exposure can be performed using biomonitoring, that is the measurement of a chemical or its metabolite(s) in easy accessible specimens of exposed individuals. Biomonitoring integrates all sources of exposure and all exposure routes (Angerer et al., 2007; Berode et al., 2011), therefore allowing the assessment of aggregate exposure. In the case of organophosphorus pesticides, it has been recently applied to estimate daily intake and to perform risk assessment (Katsikantami et al., 2019). However,

conventional biological monitoring of pesticide exposure may provide unrepresentative data, when the determination of indicators (often pesticide metabolites in urine) is carried out using extemporaneous samples collected at the end of a single application; this approach typically takes into account short-term exposure, disregarding cumulative and multiple exposure during the application season.

Hair has been proposed as a non-conventional matrix for biological monitoring of pesticides since the late '90s. The most interesting feature of hair is the wide time window for detection, therefore integrating cumulative exposure happened during months prior of the sampling (Pragst and Balikova, 2006). During the hair growing phase the cells in the bulb divide rapidly, forming a thin filament, with elongation rate of about 1 cm every 28 days. The richly vascular dermic tissue of the bulb nourishes the hair, and, at the same time, releases foreign substances contained in the bloodstream, that can be incorporated within the hair stem. Moreover, chemicals from the surrounding tissues can be incorporated into the stem. Finally, substances in the secretions of nearby sweat and sebum glands, as well as environmental pollutants, may deposit onto the hair shaft. The molecular mechanism of incorporation from blood into hair cells has been partially investigated only for drugs (Appenzeller et al., 2017). The incorporation is controlled by physico-chemical properties: lipophilic and uncharged organic molecules can easily penetrate membranes and diffuse according to the concentration gradient; conversely, for hydrophilic molecules and organic ions the membranes represent a barrier. In particular, basic drugs have significant affinity for intracellular space, leading to their accumulation in the hair; conversely, acidic drugs are found in very low concentrations in hair, due to higher affinity for the extracellular fluid (Pragst and Balikova, 2006).

Although a few studies were published so far on the measurement of persistent pesticides in the hair, such as DDT and its metabolites, mostly in the general population (Covaci et al., 2008; He et al., 2017; Raeppel et al., 2016; Tsatsakis et al., 2008), limited information is available about

93 exposure to currently used pesticides, including farmers and the general population (Lehmann et al.,
94 2018; Ostrea et al., 2014), and their accumulation during the growing season (Mercadante et al.,
95 2013; Schummer et al., 2012). Moreover, no attempt to associate the amount of pesticide used and
96 the concentration in hair in humans was done.
97 Aim of the present work was the application of hair biomonitoring to investigate aggregate and
98 cumulative exposure to several pesticides applied in the vineyards during the growing season in
99 agricultural workers (AW), in agricultural worker relatives (AR), typically residing in the rural area
100 surrounded by crops, and in the research staff personnel (RS), involved in the field study. Subjects
101 were investigated collecting information about the use of pesticides during the season and hair
102 samples before (PRE-EXP) and after (POST-EXP) the application season. This study extends a
103 previous investigation in which only penconazole and tebuconazole in hair were monitored
104 (Mercadante et al., 2018).
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2. MATERIALS AND METHODS

2.1 Study population

The study was conducted in 2012 in the vineyard areas of the provinces of Bergamo and Sondrio, Lombardy, Italy. A description of study subjects and study design was previously published (Mercadante et al., 2018). Briefly, 29 subjects were investigated, including 20 agricultural workers (AW), 4 agricultural worker relatives (AR), who lived in the area where pesticide were applied and 5 research staff members (RS), involved in sample collection during the application season. Among AW, 10 workers were farm owners and 10 workers were engaged as employees in large farms. The treatment of vineyards was performed from May to September. Hair samples were collected in April, before the application season (PRE-EXP) and again in September, at the end of the application season (POST-EXP).

A lock of hair was cut, as close as possible to the root, in the occipital region of the head, using fine scissors; the lock had a diameter of approximatively 5-8 mm and a variable length. The lock of hair was attached with paper masking tape on a sampling sheet that indicated the direction of the hair (root-tip) and were stored at room temperature in the dark. Subjects were instructed to keep a lock of occipital hair in case of haircut between PRE- and POST-EXP sampling. For this reason, they were equipped with a hair collection kit, including sampling instruction and a collection sheet.

A questionnaire to collect personal information was administrated by the research staff. At the end of the season, farmers provided information about type and amount of pesticide formulations used, the number and mode of application, and the use of personal protective equipment (PPE). In particular, the name and quantity of pesticide formulations were obtained from field records, compulsorily compiled by AW when using pesticides.

All subjects were informed about the aim of the study and signed a written informed consent. The study was performed in the frame of workers' health surveillance, under the supervision of sanitary personnel, according to the Italian law (Decreto Legislativo 9 aprile 2008).

2.2 Chemicals

The list of measured pesticides, together with CAS number, their agrochemical category, and their approval status according with the EU regulation (European Commission; National Center for Biotechnology Information), is reported in Table 1. Analytical reference standards of pesticide and their isotopically labeled internal standards were purchased through Sigma-Aldrich (Zwijndrecht, Netherlands), HPC (Cunnersdorf, Germany), and TRC (Toronto Research Chemicals, Canada). In case of neat solid standards, stock solutions were prepared in either methanol, acetonitrile or acetone at 1,000 or 2,000 µg/ml (for details see Supplemental Information). A mix stock solution of the pesticides and a mix stock solution containing all internal standards (50 ng/ml) were prepared in acetonitrile. Acetonitrile, methanol and LC-grade water were purchased from Actu-all Chemicals (Oss, the Netherlands); formic acid and ammonium formate from Merck (Darmstadt, Germany).

2.3 Hair preparation and extraction

Sample preparation was performed as described before (Mercadante et al., 2018; Polledri et al., 2018). Briefly, a segment of hair lock, with an approximate length of 5 cm and a weight of 50-100 mg was placed in a glass vial. This segment was a PRE-EXP hair sample, or a POST-EXP hair sample or a sample self-collected, in case of haircut during the application season. Water (2 ml) was added to the sample and the contaminants on the surface removed by vortexing at room temperature for 60 s. After rinsing, the aqueous solution was removed using a glass pipette, and the hair was dried at 60°C for one hour. Hair sample was cut into small pieces with metal scissors, and introduced into a cryogenic tubes (Eppendorf, Safe-Lock tube, Milan, Italy) containing three steel balls. The tube was placed in a grinding jars and cooled down with liquid nitrogen for about 15 min; then the sample was milled using a ball mill (MM400, Retsch Italy, Torre Boldone, Italy) operating at frequency of 25 Hz for 2.5 min. About 50 mg of hair powder were transferred into a glass vial and extracted with 2 mL acetonitrile at 45°C with a horizontal shaker with a rotatory vibration, operating at 150 rpm, for 3 hours. To 250 µl of the extract, 6 µl of a mix solution of isotopically

labelled internal standards was added (see Supplemental material). The extracts were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

2.4 LC-MS/MS analysis of the pesticides

Twenty-seven pesticides were analyzed; of these 14 were ingredients of formulations actually applied in vineyards by one or more AW, other 13 pesticides were included as possible environmental contaminants or from dietary exposure.

The LC-MS/MS system consisted of an injection and pump system and column oven from Waters (Acquity, Ettenleur, the Netherlands) and a Xevo TQS triple quadrupole mass spectrometer (Waters) equipped with an ESI source operated in positive mode. A volume of 10 µl of the extract were injected into the LC-MS/MS system. For LC separation, a 100 mm × 2.1 mm ID, 1.8 HSS T3 from Waters was used. The LC mobile phases were water (A) and methanol/water 95/5 (B) both containing 5 mM ammonium formate and 0.1% formic acid. The LC eluent gradient was as follows: 1 min isocratic at 100% A, then a linear gradient to 45% B at 2.5 min, followed by a linear gradient to 100% B at 8.5 min. For complete elution of all matrix co-extractants from the column, the final composition was held for 3 min. In 0.5 min the initial conditions were restored and then equilibrated for 2 min before the next injection. The LC flow rate was 400 µl/min. The temperature of the column oven was 50°C. Measurements were performed in MRM mode. Electrospray ionization conditions were set as follows: capillary voltage 3,000 V, source offset 50 V, source temperature 150°C, desolvation temperature 450°C, cone gas 150 L/h, desolvation gas flow 800 L/h. Pesticide specific acquisition details are provided in the Supplementary information. Data processing was done using MassLynx.

Together with the hair extracts, calibration solutions in solvent were analyzed (0.25, 0.63, 1.25, 1.88, 3.75, 6.25 ng/ml) at the start and end of the sequence. Calibration solutions and extracts contained isotopic labels at 1.2 ng/ml. For quantification, the peak areas of the quantifier transition was normalized against the peak area of the corresponding isotopically labelled internal standard

(no normalization was done for 5 pesticides for which the label was not available). The concentrations in the extracts were determined using the calibration equation obtained by linear regression, after which conversion to pg/mg hair was done taking into account the weight of the hair and extract concentration.

Limits of detection (LOD) of the investigated pesticides were estimated based on a signal-to-noise of 3. For this, peak heights of pesticides present in the hair extracts in the lower range (1-5 pg/mg) were used. The LODs were in the range 0.5-2 pg/mg, except for imidacloprid for which a signal in the blank occurred and an elevated LOD of 15 pg/mg had to be used. The individual LODs are included in Table 1.

2.5 Statistical analysis

The concentration of pesticides in hair was classified as detectable or not detectable, based on the comparison with LOD of the assay. Six pesticides, namely dimethenamid, fluopyram, prochloraz, propamocarb, chloridazon and cyhalothrin, that were not applied by AW and were never detected in any hair sample, were not further considered in statistical analysis.

For the other 21 pesticides, we reported either the number (%) of samples with pesticide levels >LOD or the median (min-max) levels restricted to samples with concentrations >LOD.

To compare frequencies of detectable samples across study groups, the Fisher's exact test was used. Concentrations of pesticides in hair were not normally distributed; therefore, to compare levels in the study groups, the Kruskal-Wallis test was used.

In order to evaluate (POST-EXP – PRE-EXP) differences, for each pesticide we calculated the difference between frequencies of detection. For detectable samples only, the log-difference of concentrations and then the geometric means (GM) of concentration differences were calculated. For both frequency differences and GM of differences, we provided 90% confidence intervals (90% CI) (Consonni and Bertazzi, 2017; Sterne and Smith, 2001). We preferred to work with geometric means because calculation of CIs is straightforward (while for medians there are different methods

for calculating CIs); in any case, medians and geometric means were highly correlated (Spearman correlation coefficient: 0.923). Both before-after detection frequency differences and GM of differences were visualized in forest plots.

The correlation between the median amount of each applied pesticide and its median level in the hair of farmers was performed on log-transformed variables by calculating the Pearson's r coefficient and by fitting a linear regression model.

Statistical analysis was performed using the SPSS 25.0 package for Windows (SPSS Inc., Chicago, IL, USA) and Stata 15 (StataCorp. 2017). Forest plots were produced with the Stata "metan" command.

3. RESULTS

3.1 Study population and hair samples

In Table 2 selected characteristics of study subjects and hair samples are summarized. 29 subjects were included in the study, of which 20 AW, 4 AR and 5 RS. AW were 100% males, RS were mostly males (80%), while AR were mostly females (75%). The median age was similar in AW and AR, while RS were younger. From study subjects 22 PRE-EXP hair samples, 8 hair samples self-collected during the application season, and 25 POST-EXP hair samples, were obtained. Pair samples, i.e. samples obtained from the same individuals in PRE-EXP and POST-EXP sampling, were 18; 12 pairs were from AW, 2 pairs were from AR, and 4 pairs were from RS.

3.2. Records on the use of pesticides and correlations

In Table 3 some information about the use of organic pesticides, obtained from records for 16 AW for which the POST-EXP hair samples were available, and median concentrations of pesticides in detectable samples, are summarized.

Applied pesticides were 14, of which the most frequently used were dimethomorph (94%), penconazole (87%), cyazofamid (75%), fenamidone (69%) and quinoxyfen (69%); among all pesticides, the mean of total amount applied during the entire spray season, eventually as a sum of multiple applications, ranged from 12 to 7200 g. They were all fungicides, with the exception of chlorpyrifos, that is an insecticide.

Among applicators, in POST-EXP hair samples the frequency of detection was 100% for the majority of pesticides, with the exception of cyazofamid (1 positive sample out of 12 users), fenamidone (10 positive samples out of 11 users) and imidacloprid (no positive sample out of 1 user); the median levels ranged from 1.5 pg/mg hair for cyazofamid to almost 2000 pg/mg hair for boscalid.

In AW we found a substantial linear correlation, with Pearson's r = 0.682, between levels of pesticides in hair and the corresponding total amount of pesticides applied (both log-transformed)

(Figure 1), eventually as a sum of multiple applications. From the regression equation [log(hair) = 12.9 + 0.17 x log(amount applied)] we calculated that, for a tenfold increase of applied pesticide, there was an average increase in hair concentration of $(10^{0.17} - 1) \times 100 = 47.9\%$.

3.3 Pesticides in hair

Results of 21 pesticides detected in hair of study subjects, among the 27 pesticides investigated, of which the first 14 were contained in the formulations applied by AW, are reported in Table 4.

Results are divided by sampling time and by study group. Data are given as the number and the percentage of detectable samples, and median, minimum and maximum concentrations. For the eight subjects who performed haircuts during the application season, the POST-EXP results are given as the sum of pesticide levels found in POST-EXP sample and in the intermediate self-collected sample. We recognize that this is a non-ideal sampling mode; it was adopted to let agricultural workers keeping their usual habits and avoiding too many constrains that would have discouraged them from participating in the study.

Considering PRE-EXP samples, in AW, 17 out of 21 pesticides were detectable at least in one subject; conversely, in AR and RS only 7 and 4 pesticides out of 21 pesticides were detectable at least in one subject. In detectable samples, the median levels of pesticides in hair was in the low pg/mg hair; AW showed the highest values, with concentration ranging from 1.9 to 42.2 pg/mg hair.

Considering POST-EXP samples, in AW all pesticides applied were detectable at least in one subject; while in AR and RS 14 and 9 pesticides out of 21 were detectable at least in one subject. The comparison among frequency of detection in study groups showed the highest detection frequency in AW. In positive samples, the median levels of pesticides in hair was in the low pg/mg hair, with few exception for dimethomorph, chlorpyrifos, metrafenone, boscalid and pyrimethanil in both AW and AR, and cyprodinil, penconazole, and quinoxifen in AW, for which tens pg/mg were found. In RS sporadic detectable samples were found, with the highest median level for

dimethomorph. The comparison among median levels of hair pesticides in study groups showed the highest levels in AW for dimethomorph, penconazole and quinoxyfen.

In all groups the percentage of detectability considerably increased from PRE-EXP to POST-EXP samples.

In subjects for which both PRE and POST-EXP samples were available (N pairs = 18), we found positive differences between the frequency of detection for the majority of pesticides (one among those applied and three among not applied) (Figure 2).

In all groups, the concentration of pesticides in hair generally increased from PRE-EXP to POST-EXP samples. Several differences were found in AW, with higher values in POST-EXP samples (Table 4).

In subjects for which pesticides were detectable in both PRE and POST-EXP samples (N pairs from 3 to 14, depending on the pesticide), all geometric means of differences were on the positive side (Figure 3).

4. DISCUSSION

The present study enlarges the determination of pesticides in hair of vineyard AW, AR and RS, already part of a previous study focused on fungicides penconazole and tebuconazole (Mercadante et al., 2018), to 27 pesticides. The presence of the majority of the applied pesticides in the post-season hair samples, the difference between pre- and post-season frequency of detection and level of many pesticides, and finally, the proportional increase of hair levels with the increasing quantity of applied pesticides, strengthens the use of hair as a matrix for biomonitoring cumulative and aggregate exposure.

Hair biomonitoring is particularly interesting, as hair is a non-invasive matrix in which chemicals can accumulate along time, potentially allowing an enlargement of the detection window in term of both time and number of toxicants. It may affect environmental epidemiology as health effects following exposure to toxicants are associated with low and multiple/continuative exposures. The present European Union regulatory system authorizes the commercialization and use of pesticides only when they do not negatively impact the health of human and the environment (European Commission, 2009). For this reason, persistent and bio accumulative pesticides, such as DDT and other chlorinated chemicals, were banned. Consequently, plasma and/or breast milk, specimens used to evaluate past exposure to persistent pesticides (Bevan et al., 2017; van den Berg et al., 2017), may not be suitable for pesticides in use nowadays; conversely, hair should be taken into consideration. In the present work pesticides measured in hair samples were all authorized at the time of the study (Table 1).

A further interesting feature of hair biomonitoring is the possibility of measuring pesticides themselves, disregarding metabolism. In fact, human metabolites of pesticides are often unknown, as biotransformation studies, carried out in the frame of the pesticide authorization process, are typically performed by oral dosage of pesticides in experimental animals. We recently showed that the main metabolite of the fungicide penconazole in human, dermally exposed in agriculture, is the

hydroxyl derivative PEN–OH (4-(2,4-Dichloro-phenyl)-5-[1,2,4]triazol-1-yl-pentan-2-ol) (Mercadante et al., 2016); this is different from the carboxylic acid derivative PEN-COOH (4-(2,4-Dichloro-phenyl)-5-[1,2,4]triazol-1-yl-pentanoic acid) previously identified in experiments performed in rodents and goats (WHO/FAO, 2016). Besides, the discovery of metabolites relevant for humans and their measurement for biomonitoring purposes requires analytical assay development, including the availability of pure compounds for the quantitative determination. These studies are costly and often require a custom synthesis of chemicals. On the contrary, analytical grade pesticides and their isotopically labelled internal standards are commercially available; moreover, analytical assays for the determination of pesticides in several matrices, such as water, soil, and food, are available as well, limiting the analytical work for measuring pesticide in hair to the adaptation of existing assays. This approach was followed in the present work, for the determination of 27 chemicals (Mol et al., 2016; Tienstra and Mol, 2018).

Considering farmer's records, the total number of applied pesticides was 42. However, not all were measurable in hair by LC-MS/MS. In fact, the most abundant pesticides were elemental sulphur and copper sulfate, both inorganic chemicals. Among the applied pesticides, 14 nonionic organic chemicals were assayed. In applicators, the detection frequency was almost 100% for 12 pesticides, but cyazofamid, for which only 1 applicators out of 12 had detectable amount in hair, and imidacloprid, not detected in the single user (Table 3). While for imidacloprid, this is probably attributable to the small amount of the applied pesticide (12 g) and the high LOD of the assay (15 pg/mg hair), for cyazofamid the same arguments cannot be advocated, given the larger applied quantity (313 ± 297 g) and the lower LOD (1 pg/mg hair). Possibly, physico-chemical properties of this pesticide can explain the result.

Notwithstanding the mechanism of incorporation of pesticides in hair is poorly understood, the quantity of applied pesticides was positively correlated with the concentration of the hair pesticides (Figure 1). Moreover, the correlation was not improved by the introduction of the n-octanol/water

partition coefficient (K_{ow}) as independent variable in the regression analysis, suggesting that lipophilicity is not playing a relevant role in explaining this mechanism. On the other hand, this correlation is important to support hair for quantitative biomonitoring of cumulative pesticide exposure; this supports our previous result for penconazole, showing a relationship between the number of treatments during the season and the concentration of penconazole in hair (Mercadante et al., 2018). To our knowledge, this is the first example of quantitative relationship between human exposures to environmental pollutants and their concentration in hair. Previous attempts to correlate dose and hair concentration are limited to animal experiments focused on exposure to pesticides and polycyclic aromatic hydrocarbons (Appenzeller et al., 2017; Grova et al., 2018).

The design of this study, that included the collection of hair samples before and after the application season, was meant to evaluate the capability of hair to reflect cumulative exposure. This design was previously used to investigate exposure to terbutylazine, a corn herbicide, in agriculture workers and rural residents of the Po Valley (Mercadante et al., 2013), showing an increase of pesticide during the season. Moreover, in the present study, previously published results for fungicides penconazole and tebuconazole confirmed this trend (Mercadante et al., 2018). The enlargement of hair biomonitoring to 21 pesticides further supports the capability of hair to accumulate pesticides, as both the frequency of detection and the concentration in pesticides in hair increased during the application season (Table 4). This finding was stronger for AW, reflecting the direct exposure to these chemicals, but was noticed also for AR, although with weaker evidence, certainly due to their non-occupational exposure and the low number of study subjects.

Results showed in Figure 2 and 3 include only subjects for which data pairs were available. An increase in the frequency of detection was found for all 14 applied pesticide (Figure 2), with the exception of imidacloprid, applied in very low amount and detected only in one subject in both POST- and PRE-exposure hair samples. Among 7 not applied pesticide, the frequency of detection was positive for 4 pesticides, negative for two and did not change for one pesticide; the increased

352 frequency of detection for some pesticides suggests a contamination of the rural environment,
353 irrespectively from a direct handling. In fact, study vineyards are located in hill and foothill areas,
354 neighboring other crops such as orchards and cereals. Considering the concentration of pesticides in
355 hair (Figure 3), the number of paired samples useful for the comparison between POST- and PRE-
356 exposure hair samples was variable and much lower than 18, i.e. the number of data pairs available
357 for the frequency analysis; this was because only pairs with detectable concentrations of pesticides
358 could be included. Therefore, data for 8 out of 14 applied pesticides and data 1 out of 7 not applied
359 pesticides were included; for these there were only from 3 to 14 data pairs available for the
360 comparison. Nevertheless, the analysis showed that for all applied pesticides there was an increase
361 in POST-EXP samples after the application season.

362 In the last decade, a few studies investigated pesticides in hair in both the general population,
363 including children, and agricultural workers, were performed (Yusa et al., 2015). Recent studies
364 developed assays and/or applied assays for measuring mixture of pesticides presently in use (Ostrea
365 et al., 2011; Raeppel et al., 2016; Salquèbre et al., 2012). This trend is relevant as exposure to these
366 pesticides occurs to mixture, rather than to single substances. Among previous studies, the work of
367 Schummer et al., 2012, is the most similar to ours; in 18 cereal, potato and vineyard agricultural
368 workers they measured 50 pesticides in hair; for pyrimethanil, cyprodinil, penconazole,
369 tebuconazole, azoxystrobin, pyraclostrobin, and metolachlor in post exposure hair samples, the
370 frequency of detection, from 6 to 72%, and concentration, from few to tens pg/mg hair, were very
371 similar to those found in the present study (Schummer et al., 2012).

372 Limitations of the present study are: the small number of investigated subjects, already discussed
373 in the previous paper (Mercadante et al., 2018), and the lack of reference values, useful for the
374 interpretation of detected levels of pesticides in hair. It should be recognize that the use of hair for
375 the biological monitoring of exposure to pesticides is still at its initial stage. It can be foreseen that
376 the use of new technologies, such as mass spectrometry, will be beneficial to collect additional data

for building a frame for the future interpretation of hair biomonitoring. Another limitation is the use of the LOD instead of the limit of quantification (LOQ), as the minimum concentration useful for the quantification of pesticides. This approach was adopted to increase the number of quantifiable samples, although with a consequent reduction of accuracy and precision of data; we believe that the explorative nature of our study justifies the adopted strategy.

5. CONCLUSION

Our results indicate that the determination of pesticides in hair is useful for biomonitoring mixture exposure to pesticides during the application season in agricultural workers and agricultural relatives; our results reinforce the use of hair for a quantitative biomonitoring of exposure to pesticides.

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FIGURE LEGENDS

Figure 1. Scatter plot and linear regression line between median concentration of pesticides in POST EXP hair sample of agricultural workers and the median quantity of pesticides applied during the season.

Figura 2. Difference (90% CI) between the frequencies of detection of pesticides in hair collected post exposure vs. pre exposure in all study subjects. N pairs indicates the number of paired samples available.

Figura 3. Geometric mean difference (90% CI) between the levels of pesticides in hair collected post exposure vs. pre exposure. N pairs ≥ LOD indicates the number of paired detectable samples available.