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Multi-biomarker responses to pesticides in an agricultural population from Central Brazil

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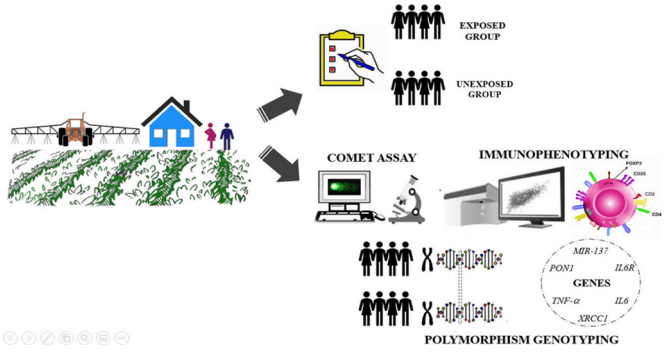
HIGHLIGHTS

- Genotoxic effects of a complex mixture of pesticides were observed in farmworkers.
- Immune changes may reflect an immune activation in defense against pesticides.
- Allele A of the TNF-α (rs361525) increased DNA damage of the farmworkers.

GRAPHICAL ABSTRACT



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ABSTRACT

We evaluated farmworkers exposed to pesticides and individuals with no history of occupational exposure to pesticides. It was performed the comet assay to evaluate DNA damage. The immunophenotyping of $TCD4^+$ lymphocyte subpopulations in peripheral blood was performed by flow cytometry. The single nucleotide polymorphisms (SNPs) in PON1, XRCC1, IL6, IL6R, TNF-α, and MIR137 genes were evaluated by real-time PCR. The exposed group was composed mostly by males (69.44%), with direct exposure to pesticides (56%) and with an average age range of 46 ± 13.89 years, being that 58.3% of farmworkers directly exposed to pesticides and reported the full use of personal protective equipment (PPE). DNA damage was greater in the exposed group ($p < 0.05$), reinforced by the use of PPE to denote a lower degree of DNA damage ($p = 0.002$). In this context, in the exposed group, we demonstrated that the use of PPE, age, gender and intoxication events were the variables that most contributed to increase DNA damage ($p < 0.0001$). Besides, the exposed group showed a significant increase in the subpopulations of T lymphocytes $CD3^+CD4^+$ ($p < 0.05$) and $CD3^+CD4^+CD25^+$ ($p < 0.0001$) and a significant decrease in $CD3^+CD4^+CD25^-FOXP3^+$ ($p < 0.05$). SNPs in the TNF-α (rs361525) gene presented a difference in the genotype distribution between the groups ($p = 0.002$). The genotype distribution of TNF-α (rs361525) was also positively correlated with the DNA damage of the exposed group ($r = 0.19$; $p = 0.01$), demonstrating a higher risk of DNA damage in the farmworkers presenting the A mutated allele. Our findings

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demonstrate that pesticides can exert various deleterious effects on human health by damaging the DNA as well as by influencing the immune system in the case of both direct or indirect exposure and these issues are associated to age, gender, intoxication and the nonuse of PPE.

1. Introduction

Pesticides have been extensively used globally to increase crop production and quality through controlling pests and for vector-borne diseases. It is noteworthy that Brazil is one country that most consume pesticides in the world (Marcelino et al., 2019; Nascimento et al., 2020; Paumgartten, 2020). Only in 2019, 439 products were authorized, the most significant number in the last 10 years (Brazilian Agriculture Minister, 2019; Nascimento et al., 2020) and even with the COVID-19 pandemic, the Brazilian government released 150 new pesticides this year (until may). Thus, almost all individuals are exposed to relatively low doses of pesticides due to environmental contamination or intentional use. According to Damalas and Eleftherohorinos (2011), at low doses of exposure pesticides do not produce any permanent harmful effects on humans.

In this context, agricultural workers and their families and individuals who reside close to fields where pesticides are applied are considered to be the group that will receive the most considerable exposure at the highest risk for adverse health outcomes (Gangemi et al., 2016; Docea et al., 2017; Jacobsen-Pereira et al., 2018; Godoy et al., 2019; Marcelino et al., 2019). As a result, humans exposed to a complex mixture of pesticides are more likely to develop different diseases due to deleterious effects on immune, hematological, nervous, endocrine, and reproductive systems (Corsini et al., 2013; Aroonvilairat et al., 2015; Campos et al., 2016; Corral et al., 2017; Docea et al., 2017; Koh et al., 2017; Aiassa, 2018; Jacobsen-Pereira et al., 2018; Godoy et al., 2019; Paumgartten, 2020).

Moreover, the development of cancer has been associated with exposure to pesticides (Gilden et al., 2010; Docea et al., 2017; Silvério et al., 2017; Jacobsen-Pereira et al., 2018; Paumgartten, 2020). Thus, biomarkers are a practical approach to assess the risk related to the use of pesticides and protect human health (Docea et al., 2017; Jacobsen-Pereira et al., 2018; Lozano-Paniagua et al., 2018; Barrón-Cuenca et al., 2019; Godoy et al., 2019). The development and validation of new and useful biomarkers to assess pesticide exposure are warranted to implement proper control measures (Araoud, 2011; Lozano-Paniagua et al., 2018).

Various *in vitro* and *in vivo* studies, as well as epidemiological approaches, have demonstrated that pesticides or their metabolites may result in genotoxic and mutagenic effects (Bolognesi, 2003; Docea et al., 2017; Kapeleka et al., 2019; Paumgartten, 2020). Therefore, the use of genotoxicity and mutagenicity biomarkers is relevant to provide early identification of biological effects (Kapka-Skrzypczak et al., 2011; Aiassa, 2018; Lozano-Paniagua et al., 2018). For assessment of mutagenic and genotoxic pesticide-induced damage, the most widely used methods are sister chromatid exchange assay, chromosomal aberrations test, single-cell gel electrophoresis (comet) assay, and micronuclei test (Aiassa, 2018; Godoy et al., 2019; Kapeleka et al., 2019; Marcelino et al., 2019).

Data from experimental and epidemiological studies have also demonstrated that exposure to pesticides can modify the immune system either morphologically or functionally contributing to the development of immune-mediated diseases, such as asthma, allergies, type 1 diabetes, thyroid diseases, rheumatoid arthritis and atherosclerosis (Gangemi et al., 2016; Requena et al., 2019; Fukuyama and Tajiki-Nishino, 2020). Regarding autoimmune diseases, Parks et al. (2011) demonstrated a higher risk of rheumatoid arthritis as well as systemic lupus erythematosus in women who self-reported the use of insecticides, with a higher risk in women reporting a farming background. Furthermore, Parks et al. (2016) observed an association between exposure to some pesticides and rheumatoid arthritis in male pesticide applicators. This same author, in another study (Parks et al., 2019), demonstrated that moderate to higher level of serum antinuclear autoantibodies are associated with the past exposure to some types of pesticides and a history of seeking medical care in male farmers occupationally exposed to pesticides.

In general, pesticides can impair immune cells function by inducing oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, disruption of the ubiquitin protease system or autophagy, and inhibition of enzymes with esterase activity (Corsini et al., 2008; Mokarizadeh et al., 2015; Fukuyama and Tajiki-Nishino, 2020). Therefore, the altered immune system may be a sensitive marker of pesticide-induced immunotoxicity, eventually affecting the development of immune-mediated disorders, and so may be predictive of eventual diseases (Corsini et al., 2013; Fukuyama and Tajiki-Nishino, 2020). Biomarkers recommended assessing immunotoxicity of pesticides include lymphocyte count, antibody-mediated immunity (serum concentrations of immunoglobulins) analysis, lymphocytes phenotypic analysis by flow cytometry, measurements of autoantibodies and markers of an inflammatory response, among others (Rojas-García et al., 2011; Parks et al., 2019; Fukuyama and Tajiki-Nishino, 2020).

It is also of particular relevance to the environmental health research to investigate Single Nucleotide Polymorphisms (SNPs) in inflammatory genes since SNPs play a critical role in the assessment of the immune response to pesticide exposure, once they can protect or increase the effects of pesticides (Araoud, 2011; Godoy et al., 2019; Teodoro et al., 2019). Individual susceptibility to develop polymorphisms can be evaluated by a wide range of genetic variations affecting critical genes involved in the metabolism process and DNA repair (Tabrez et al., 2014; Teodoro et al., 2019). For instance, the genetic variability in cytokine and microRNA genes may play a role in the risks of pesticide-related disease and can also be used as biomarkers associated with susceptibility (Gangemi et al., 2016; Sisto et al., 2019).

Regarding the metabolism genes, SNPs in cytochrome P450 (CYP), glutathione transferases (including GSTM1, GSTP1, GSTT1), acetyltransferases (NAT2), and paraoxonases (mostly PON1) genes have been widely used to evaluate interindividual differences in metabolization and detoxification of pesticides (Rojas-García et al., 2011; Teodoro et al., 2019). Additionally, polymorphisms in DNA repair genes, especially those involved in base excision repair, including OGG1 (8-oxoguanine DNA glycosylase) and XRCC1 (X-ray repair cross-complementation group 1) can be associated with higher risks of pesticide-related diseases. Moreover, gene variants have also been investigated to understand the differences in susceptibility to pesticide exposure (Tabrez et al., 2014; Teodoro et al., 2019).

Hence, the current study evaluated the impact of pesticide exposure on the health of rural workers in the southeast and southwest of Goias, Brazil, using genotoxicity, immunotoxicity, and susceptibility tests. This is a pioneer study in Central Brazil involving genetic and immunological biomarkers to identify how pesticides could impair such systems increasing the susceptibility to the development of chronic issues problems.

2. Material and methods

2.1. Study population and data collection

We carried out a cross-sectional study conducted in three Brazilian municipalities: Silvânia, Jataí and Montividiu (Fig. 1), which presents

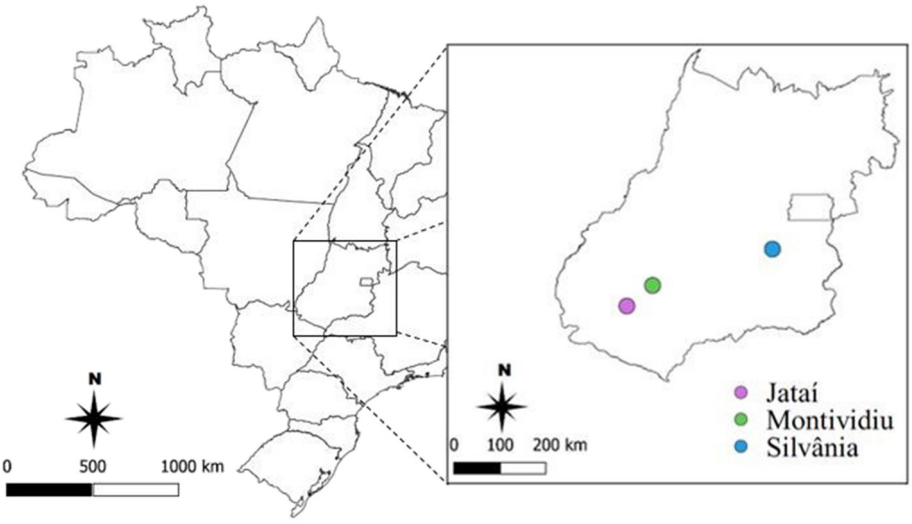

Fig. 1. Municipalities from Central Brazil evaluated in the present study.

intense agricultural activity (mainly soybean and corn crops). One hundred and eighty individuals, 125 men and 55 women, were classified as an exposed group. This group was composed of 100 individuals directly exposed to pesticides (occupationally exposed to various pesticides during storage, mixing, loading, and pesticide spraying activities), and 80 subjects indirectly exposed, living nearby crops, therefore, environmentally exposed to pesticides. The control group consisted of 180 individuals, 125 men, and 55 women, with no direct contact or closer exposure to pesticides, and were matched with the exposed group by age, gender, and lifestyle.

Sociodemographic data (age, gender, smoking habit and alcohol consumption), medical and occupational history (pesticide exposure, pesticide brand names, type of pesticides used and the use of personal protection equipment - PPE) were collected by applying a questionnaire with open-ended and closed questions from all participants. We interviewed the individuals about health issues affecting the immune system, such as: type 1 diabetes mellitus; systemic lupus erythematosus; rheumatoid arthritis; Crohn's disease; multiple sclerosis; Hashimoto's thyroiditis; Myasthenia gravis and Sjogren's syndrome. We excluded from the study in the presence of any autoimmune condition.

All participants were fully informed about the procedures and the aims of the study as well as signed informed written consent before participation. We obtained approval for this study from the Research Ethics Committee of the Federal University of Goias (reference number 2.648.494). All research procedures were according to the principles of the regulatory guidelines and standards described in Resolution No. 466/12 of the National Health Council, which approves the regulatory guidelines and standards for research involving human beings in Brazil.

A total volume of 15 mL of peripheral blood was obtained from exposed and non-exposed participants in EDTA (ethylenediaminetetraacetic acid) vacuum tubes. Samples were transferred at 4 °C to the Mutagenesis Laboratory of Federal University of Goias, and were processed, frozen, and stored at −20 °C until analysis.

It is worth mentioning that we sampled the total blood of the rural workers during the midseason and at the end of a week of application, once we could verify how exposure to such products altered the response of multiple biomarkers.

2.2. Biomarker of DNA damage

We evaluated DNA damage by alkaline single cell gel electrophoresis (comet assay) according to Singh et al. (1988), with a slight modification, mainly in the stained of the slides. Briefly, two slides were processed for each individual. A volume of 15 μL of whole blood was mixed with 120 μL of a 0.1% low-melting-point agarose solution at 40 °C. Then, we spread this mixture onto frosted slides precoated with 1.5% standard melting point agarose, covered with a coverslip, and stored at 4 °C for 5 min. After solidification, the coverslip was removed carefully. The slides were immersed in an ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, Triton X-100, and 10% DMSO, pH 10) at 4 °C overnight. The slides were placed in a horizontal gel electrophoresis tray with electrophoresis alkaline buffer (200 mM EDTA, 300 mM NaOH, pH 13) for 30 min to allow DNA unwinding. Electrophoresis was performed in the same alkaline buffer at 4 °C for 30 min in the dark (25 V, 300 mA). Afterward, 0.4 M Tris (pH 7.5) was applied to neutralize the slides three times, for 5 min. The slides were dried after fixing in absolute ethanol. In the end, DNA was stained with 100 μL of the SYBR® Green I in TE solution (50 mM Tris-HCl, 500 mM EDTA, pH 7.5) and examined under a fluorescence microscope (Axio Imager 2®, Carl Zeiss) linked to the Comet Imager version 2.2 software (MetaSystems GmbH). DNA damage was measured based on the percentage of DNA in the tail (% DNA), according to Sunjog et al. (2013). One hundred randomly selected nuclei were visualized using the 20× objective per individual.

2.3. Biomarkers of immune dysfunction

The percentages of different $CD4^+$ lymphocyte subsets were analyzed by flow cytometry (FACS Canto II, BD Bioscience, San Jose, CA, USA). Peripheral blood mononuclear cells (PBMC) were isolated using density-gradient centrifugation with Ficoll-Paque™ Plus (density 1.077 g/mL, GE Healthcare, Uppsala, Sweden) following the manufacturer's instructions. Briefly, ten milliliters of well-mixed whole blood were diluted 1:1 with phosphate-buffered saline (PBS) pH 7.4. For each blood sample, 3 mL of the Ficoll-Paque PLUS (GE Healthcare) were placed into the bottom of the 15 mL centrifuge tubes, and then diluted blood was carefully placed over the Ficoll-Paque layer. The tubes were centrifuged at 1500 rpm for 30 min at 4 °C. After centrifugation the red blood cells (erythrocytes) as well as the polymorphonuclear cells are located at the very bottom of the 15 mL tube (red layer), followed by the ficoll layer over it (clear layer). Between the ficoll and the plasma layers, there is the ring (white layer) of peripheral blood mononuclear cells (PBMCs) which one was carefully collected by using a Pasteur pipette into a new tube containing PBS pH 7.4. Tubes were centrifuged for 10 min at 1500 rpm (the procedure was performed twice). Finally, the supernatant was discarded, and 1 mL of PBS pH 7.4 enriched with 1% of bovine fetal serum (BFS) was added to the pellet. In a Neubauer counting chamber, we counted viable cells using Trypan

blue 1: 9 (10 μL of the sample in 90 μL of Trypan blue) and one million cells were aliquoted to receive the mix of antibodies and a small amount were aliquoted to be the unstained sample (control). This control tube is used to set up the equipment in order to distinguish exactly the cell autofluorescence from the fluorescence due the markers. The antibody mix to extracellular staining was composed by BV480-conjugated anti-human CD3, PerCP-conjugated anti-human CD4, PECy7-conjugated anti-human CD25 (BD Biosciences – San Diego, CA, USA). PBMC received 3uL of antibodies mix and were incubated at 4 °C for 20 min in the dark. Then, cells were washed with 1% BFS-PBS and permeabilized in a fixation/permeabilization buffer for 18 h at 4 °C in the dark. After incubation, cells were stained intracellularly for Alexa Fluor 647-conjugated anti-human FoxP3 at 4 °C for 20 min in the dark. After, the cells were washed twice in 1%BFS-PBS and analyzed using BD FACSDiva™ software (BD Biosciences, USA). At least 10,000 lymphocytes for each sample were acquired and analyzed.

2.4. Biomarkers of susceptibility (SNPs)

Genomic DNA was extracted from whole peripheral blood using the ReliaPrep™ Blood gDNA Miniprep System extraction kit (Promega®, USA), according to the manufacturer's protocol. Then, the DNA samples were quantified using a NanoVue Plus™ spectrophotometer equipment (GE Healthcare, USA) following the manufacturers recommendations. The DNA purity of all samples was 1.8 (according to 260/280 nm). The 7 selected SNPs (rs662, rs25487, rs1800795, rs228145, rs361525, rs1799964 and rs1625579) were genotyped using the TaqMan® SNP Genotyping Assays C___2548962_20, C____622564_10, C___1839697_20, C__16170664_10, C___2215707_10, C___7514871_10 and C___8946584_20, respectively. All primers and probes were designed by Thermo Fisher Scientific (Waltham, MA, USA), and genotyping analyses were performed on One Step Plus Real-Time PCR system (Applied Biosystems, USA) according to the manufacturer's protocol. We performed singleplex PCR reactions and for all the 7 SNPs and the amplifying reactions conditions, as follows: Genomic DNA was diluted to 10 ng/μL, and 2 μL was used for Real-Time PCR. In all, 5 μL of TaqMan™ Master Mix and 0.25 μL of TaqMan™ SNP Genotyping were added to the diluted DNA, and Milli Q water was added to make up the final volume to 10 μL. The thermocycler was set at 50 °C for two minutes and at 95 °C for 10 min to activate polymerase AmpliTaq®. In all, 45 cycles of denaturation (95 °C for 15 s) and annealing-extension (60 °C for 1 min) were used to amplify the DNA sequence.

2.5. Statistical analysis

We presented continuous variables as mean and standard deviation and categorical variables as frequencies with the corresponding percentages. We used the Kolmogorov Smirnov to test the data normality. We applied the Chi-square test for categorical variables and the Student *t*-test for continuous variables. A linear regression analysis was carried out between DNA damage and exposure time in the group composed only by farmworkers. The Mann–Whitney *U* test compared DNA damage and the proportion of lymphocyte subpopulations between groups. The association between DNA damage and the number of T cells $CD3^+CD4^+$ and $CD3^+CD4^+CD25^+$, and the *TNF-α* (rs361525) polymorphism, were tested using Spearman's nonparametric correlation. For these analysis we used the Statistica 7.0 software (StatSoft Inc., 2004).

We also carried out a generalized linear mixed model (GLMM) to estimate which predictor variable (age, gender, PPE, intoxication, exposure time, alcohol usage and smoking habit) was mostly associated to DNA damage. This analyze was carried out with the glm function (vegan package) [Oksanen et al., 2012] and using the Poisson distribution. The significance assessment was performed based on a null model and considering ΔAIC.

The estimation of genotype and allele frequencies was carried out by direct counting. Allele and genotype frequencies were compared between pesticide-exposed individuals and controls by the $\chi 2$ test. We also tested Hardy-Weinberg Equilibrium (HWE). The Hardy-Weinberg equilibrium is considered the null model of Population Genetics and serves to test hypotheses about the change in allele and genotype frequencies in populations. In an infinite population, in which matings occur at random and in the absence of evolutionary factors such as natural selection, mutation and gene flow, the allelic and genotype frequencies remain constant throughout the generations, and are, respectively, p and q and p^2, 2pq and q^2 (Mayo, 2008). These analysis were carried out with Genepop v. 1.2 (Raymond and Rousset, 1995). The p-value was between 1 and 5% ($p < 0.01$ or <0.05, respectively).

3. Results

3.1. Sociodemographic and occupational characteristics

The demographic characteristics of the study group are shown in Table 1. The exposed and control groups did not differ regarding age, gender, smoking habits, and alcohol consumption. The time of exposure of the exposed group presented a median of 16.3 ± 10.3 years. The use of PPE was described by 58.3% of the farmworkers, while 41.7% reported not using it at all. A total of 37 individuals of the exposed group (20.6%) reported acute pesticide poisoning consisting of 10 indirectly exposed subjects (27%) and 27 directly exposed individuals (73%). Of the 27 directly exposed rural workers who were intoxicated, six of them did not use PPE. One hundred (87 men and 13 women) were individuals directly exposed to pesticides and 80 subjects (38 men and 42 women) live nearby crops and, therefore, were environmentally exposed to pesticides.

The most frequently self-reported chronic health problems among exposed individuals were high blood pressure (18.9%), allergy (17.2%), diabetes type 2 (11.1%), and thyroid disease (4.4%). Regarding pesticides and in accordance to the organism they kill (Megha et al., 2018), exposed individuals reported frequent use of herbicides (47%) and followed by insecticide (42%) and fungicide (11%). The more commonly used pesticides were glyphosate (40.9%), 2,4-D (15.6%), cypermethrin (10.2%), deltamethrin (8.1%), and atrazine (5.4%). The most common crops were soybean (48%) and corn (34%) crops.

3.2. Biomarker of DNA damage

DNA damage in exposed and controls are described in Table 2. We demonstrated more DNA damage in the exposed group compared to the non-exposed group ($p < 0.05$), independent of the type of exposure (if direct or indirect Table 2). No significant difference in the DNA damage was observed based on smoking habits and alcohol consumption ($p > 0.05$). Age, gender distribution, the use of PPE and intoxication events showed statistically significant differences in DNA damage

Table 1
Socio-demographic and lifestyle variables from the study population.

Variable	Groups		p-value
	Exposed (n = 180)	Non-exposed (n = 180)	
Age (years)	46.0 (±13.9)	45.9 (±14.9)	0.9[a]
Sex			
Women	55 (30.6%)	55 (30.6%)	1[b]
Men	125 (69.4%)	125 (69.4%)	
Smoking habits			
Yes	33 (18.3%)	35 (19.4%)	0.8[b]
No	147 (81.7%)	145 (80.6%)	
Alcohol consumption			
Yes	90 (50%)	75 (41.7%)	0.1[b]
No	90 (50%)	105 (58.3%)	

[a] p value associated to Student's t test.
[b] p value associated to chi-square test.

Table 2
Mean and standard deviation of comet assay parameter (percentage of DNA in tail) for the study population, regarding general characteristics.

Variable	Parameter of DNA damage (mean ± standard deviation)	
	Exposed (n = 180)	Unexposed (n = 180)
	% DNA[b]	% DNA
	18.4 ± 8.1	15.8 ± 7.7 ($p = 0.004$)[a]
Exposure		
Directly exposure (n = 100)	17.9 ± 8.0	–
Indirectly exposure (n = 80)	19.1 ± 8.1 ($p = 0.3$)[a]	–
Sex		
Women	21.7 ± 7.8	16.0 ± 9.1
Men		
Smoking habits	17.0 ± 7.8	15.8 ± 7.1
Yes	$p = 0.1$[a]	$p = 0.4$[a]
No	16.3 ± 6.8	16.9 ± 6.7
Alcohol consumption	18.9 ± 8.3	15.6 ± 8.0
Yes	$p = 0.1$[a]	$p = 0.7$[a]
No	17.5 ± 7.9	16.1 ± 6.5
Use of PPE	19.4 ± 8.1	15.6 ± 8.5
	$p < 0.001$[a]	–
Yes	16.5 ± 7.6	–
No	21.1 ± 7.9	–

[a] p value associated to Mann-Whitney test; PPE: Personal protective equipment.
[b] Percentage DNA in tail.

($p < 0.001$), demonstrating considerable DNA lesions among older farmworkers, women, individuals that reported intoxication and who did not use PPE (Table 3). We did not find association between time of exposure to pesticides and DNA damage ($p > 0.05$).

3.3. Biomarkers of immune dysfunction

Of the 360 individuals, 173 were analyzed by flow cytometry (118 exposed and 55 non-exposed). The individuals in the exposed group showed a significant increase of $CD3^+CD4^+$ ($p < 0.05$), $CD3^+CD4^+$-$CD25^+$ ($p < 0.0001$) and a decrease in the $CD3^+CD4^+CD25^-$-$FOXP3^+$ ($p < 0.05$) lymphocytes subpopulations when compared to individuals in the non-exposed group (Fig. 2A–C). However, the percentage of natural regulatory T cells ($CD3^+CD4^+CD25^+FOXP3^+$) did not differ between the groups ($p = 0.12$) (Fig. 2D). There were no statistically significant correlations between the percentage of $TCD4^+$cells and $TCD4^+CD25^+$ cells and the percentage of DNA in tail (% DNA) in the exposed group ($r = -0.15$; $p = 0.25$ and $r = -0.14$; $p = 0.13$, respectively) (Fig. 3).

3.4. Biomarkers of susceptibility

The distributions of all genotypes were in accordance to Hardy-Weinberg equilibrium. The SNPs genotyping of PON1 (rs662), XRCC1 (rs25487), IL6 (rs1800795), IL6R (rs2228145), and MIR137 (rs1625579) revealed that there was no statistically significant difference in the genotype and allele distributions among the studied groups ($p > 0.05$) (Table 4). We also did not find that the farmers presenting mutant alleles of PON1 (rs662), XRCC1 (rs25487), IL6 (rs1800795), IL6R (rs2228145), and MIR137 (rs1625579) presented increased DNA damage (Fig. 4). However, there was a difference in the SNPs distribution of the TNF-α (rs361525) gene between the study groups ($p = 0.002$) (Fig. 4). Additionally, a significant positive correlation was found between the TNF-α (rs361525) polymorphism and DNA damage ($r = 0.19$; $p = 0.01$), but we found no correlation between TNF-α (rs361525) and the percentage of DNA in tail (% DNA) in the control group ($r = 0.05$; $p = 0.47$).

Table 3
A Generalized Linear Model (GLM) demonstrating the predictor variables most associated to DNA damage. Significance assessment was performed based on a null model and considering ΔAIC[a].

Predictor variable	Effect
PPE	0.5512693
Age	0.1340231
Gender	4.2548063
Intoxication	2.2281703

[a] ΔAIC = 6.23 (Null model = 1263.023 / Model = 1256.791).

4. Discussion

This is the first study from Central Brazil that demonstrated how genetic and immune biomarkers are associated to the exposure of a complex mixture of pesticides (either simultaneously or sequentially) in farmworkers. These individuals handled and applied the combination of different types of pesticides to decrease the number of applications, reducing costs and work hours and broadening the spectrum of pest controlled (Pedlowski et al., 2012; Marcelino et al., 2019). This habit may increase the health risk associated with exposure to pesticides. Therefore, this practice's impact has been widely explored (Paiva et al., 2011; Pedlowski et al., 2012; Hernández et al., 2017; Bernieri et al., 2019; Oliveira et al., 2019, 2019; Marcelino et al., 2019).

Besides, lack of or incomplete use of personal protection equipment (PPE) by farmworkers may potentiate the deleterious health effects in humans increasing the concentration of active chemicals in skin, mouth, eye, and respiratory tract, leading to a growth of pesticides absorption (Pasiani et al., 2012; García-García et al., 2016). Farmworkers also have poor safety and hygiene practices, such as not washing hands after pesticide application, which also can lead to adverse health effects (Damalas and Eleftherohorinos, 2011; Khanal and Singh, 2016; Godoy et al., 2019).

The non-usage of PPE during pesticide handling was also pointed out in other studies (Pasiani et al., 2012; García-García et al., 2016; Ali et al., 2018; Godoy et al., 2019; Bernieri et al., 2019). Farmworkers reported that PPE is poorly tolerated because of high cost and discomfort when used in warm weather, similar to other studies conducted in tropical areas (Recena et al., 2006; García-García et al., 2016; Caldas, 2016). The importance of using PPE was confirmed in this study, showing increased DNA damage for those who reportedly did not use them.

The consequences of the mishandling of pesticides and non-use of PPE were also reflected in the number of individuals that had acute pesticide poisoning (APP) after spraying pesticides. APP has been demonstrated in occupational and environmental exposures (Farias et al., 2009; Pasiani et al., 2012; Godoy et al., 2019; Hendges et al., 2019). In Brazil, according to the National System of Toxic-Pharmacological Information (SINITOX), 3820 cases of poisoning due to occupational exposure to pesticides were registered in 2014 (Queiroz et al., 2019; Nascimento et al., 2020) an underestimated number due to both underdiagnosing and underreporting (Caldas, 2016; Queiroz et al., 2019; Nascimento et al., 2020). In Brazil, the Decree n°. 4.074 (from January 4th, 2002) regulates the enforcement of Brazilian Pesticide Law (Federal Law n°. 7.802 from July 11th, 1989), contributed to the flexibility in the procedures involving agrochemicals, leading to higher availability and consumption with consequently increased in pesticide poisoning and populational exposure (Hendges et al., 2019).

Also, families of farmworkers are often environmentally exposed to multiple pesticides, either by living near crops or by having contact with contaminated clothes and work tools without personal protection (Damalas and Eleftherohorinos, 2011; Parks et al., 2016; Doğanlar et al., 2018). In general, farmworkers' families are exposed to lower levels but for a longer duration to pesticides. Thereby may be more vulnerable to adverse effects, especially pesticide poisoning (Ward et al., 2006; Shirangi et al., 2011; Parks et al., 2016). The chronic diseases reported by pesticide-exposed participants in our study is in agreement with studies performed in similar conditions (directly and indirectly exposition) (Mrema et al., 2017; Kongtip et al., 2018; Barrón-Cuenca et al., 2019). Moreover, other epidemiological studies have shown a higher

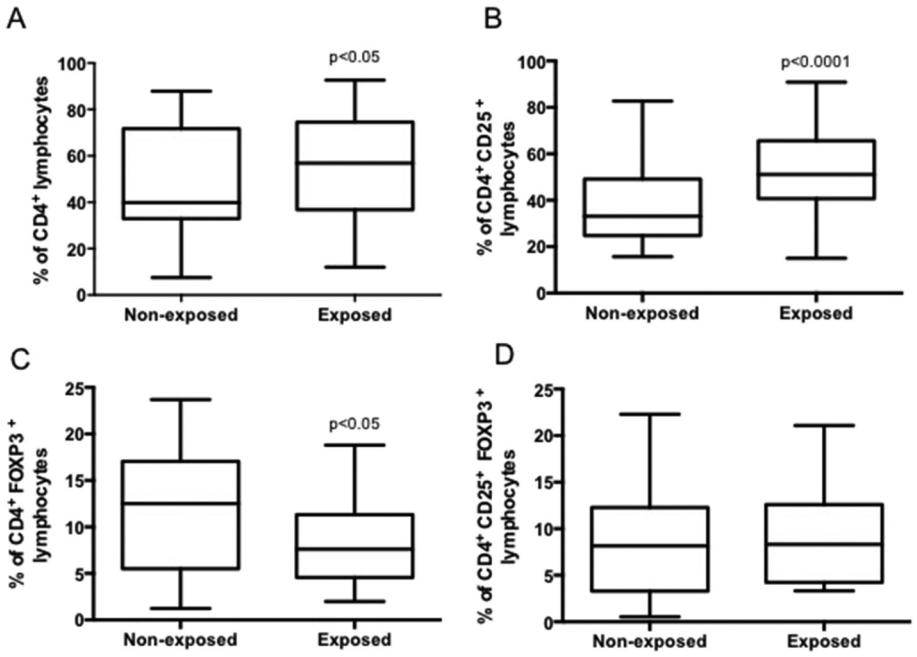

Fig. 2. Representative box plot of T cell subpopulations percentage in the peripheral blood of exposed and non-exposed individuals. A: percentage of $CD4^+$ T cells ($p < 0.05$); B: percentage of $CD4^+CD25^+$ T cells ($p < 0.001$), C: percentage of $CD4^+FOXP3^+$ T cells ($p < 0.05$), D: percentage of $CD4^+CD25^+FOXP3^+$ T cells ($p = 0.12$). For the box plot, the horizontal line represents the mean and the boxes represent the interquartile range. Student's T test was employed to analyze the data.

risk of psychiatric problems, in people exposed to pesticides – especially those who have suffered from pesticide poisoning – in different countries such as Chile (Corral et al., 2017), Egypt (Rohlman et al., 2019), Korea (Koh et al., 2017), Mexico (Serrano-Medina et al., 2019), and Brazil (Meyer et al., 2010; Farias et al., 2014; Campos et al., 2016; Nascimento et al., 2020).

Mostafalou and Abdollahi (2013) and Kim et al. (2017) concluded that the mechanisms associated with developing pesticide-related chronic diseases are challenging to elucidate due to various factors, mainly inherent genetic susceptibility to associated pesticide diseases. However, the statistical associations between exposure to certain pesticides and the incidence of some chronic diseases are compelling enough to create concern. Besides the chronic effects observed in individuals exposed to pesticides, we also observed an increase in the DNA damage of exposed individuals compared to the control group ($p < 0.05$). Previous studies on farmworkers exposed to pesticides from our group (Khayat et al., 2013; Godoy et al., 2019) and others (Marcelino et al., 2019; Barrón-Cuenca et al., 2019) showed increased DNA damage measured by the comet assay compared to the control group. As well as found in the present study, workers in the previous studies were in contact with a complex mixture of pesticides. Intranuovo et al., 2018 evaluated DNA damage in lymphocytes of agricultural workers exposed to pesticides by comet assay in a cross-sectional study in two provinces of Italy (Bari and Taranto) of the Apulia region. Those authors confirmed the genotoxicity of pesticides used by the exposed group and suggested the utility of the comet assay in the biomonitoring of occupational exposure to genotoxic agents. It is noteworthy that the reduction of molecular oxygen (O_2) during exposure to pesticides results in the production of highly reactive oxygen species (ROS), leading subsequently to oxidative damage to DNA (Kaur and Kaur, 2018).

In agreement with the gender of exposed individuals, women displayed higher levels of DNA damage than men ($p < 0.001$), probably because they are the most actively involved laborers in the crops until harvest, as reported by Rekhadevi et al., 2016 and are also responsible for washing their partners' working clothes or male relatives. Therefore, women had direct and chronic contact with pesticides and their residues.

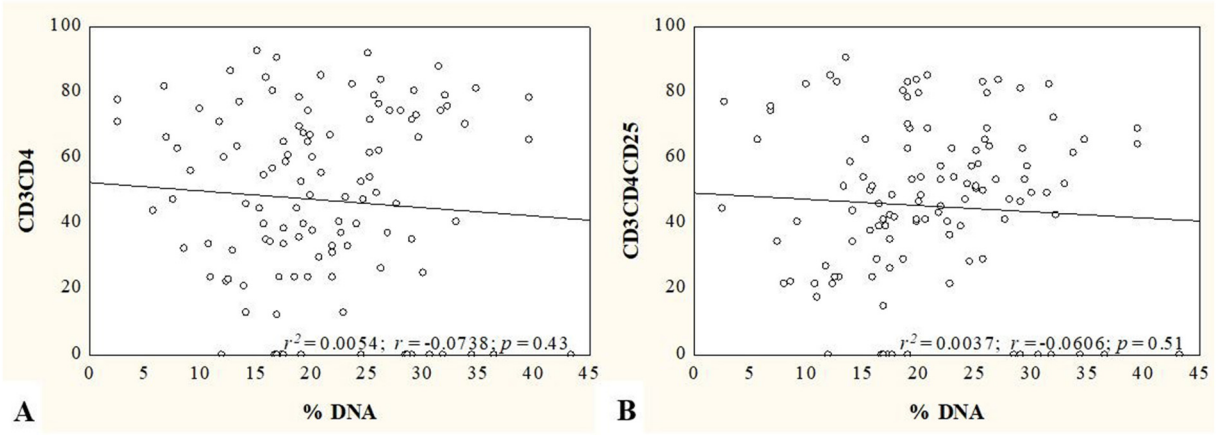

Fig. 3. Scatter plot illustrates the correlation between the percentage of DNA in tail (% DNA) and the number of T cells $CD3^+CD4^+$ (A) and $CD3^+CD4^+CD25^+$ (B) in the peripheral blood of the exposed group. Each dot represents a single individual, $p < 0.05$ (Spearman's correlation).

Table 4
Distribution of PON1, XRCC1, IL6, IL6R, TNF-α and MIR137 SNPs for the study population.

Group	Genotype			Allele frequency		p
				Wild allele	Mutated allele	
rs662 (PON1)						
	TT	TC	CC	T	C	
Exposed	61 (33.9%)	88 (48.9%)	31 (17.2%)	0.58	0.42	0.09[a]
Unexposed	71 (39.4%)	68 (37.8%)	41 (22.8%)			
rs25487 (XRCC1)						
	CC	CT	TT	C	T	
Exposed	105 (58.3%)	66 (36.7%)	9 (5%)	0.74	0.26	0.14[a]
Unexposed	92 (51.1%)	70 (38.9%)	18 (10%)			
rs1800795 (IL6)						
	GG	GC	CC	G	C	
Exposed	100 (55.6%)	66 (36.7%)	14 (7.8%)	0.7	0.3	0.09[a]
Unexposed	81 (45%)	76 (42.2%)	23 (12.8%)			
rs2228145 (IL6R)						
	AA	AC	CC	A	C	
Exposed	48 (26.7%)	70 (38.9%)	62 (34.4%)	0.46	0.54	0.79[a]
Unexposed	47 (26.1%)	76 (42.2%)	57 (31.7%)			
rs1799964 (TNF-α)						
	TT	TC	CC	T	C	
Exposed	83 (46.1%)	69 (38.3%)	28 (15.6%)	0.64	0.36	0.07[a]
Unexposed	88 (48.9%)	51 (28.3%)	41 (22.8%)			
rs361525 (TNF-α)						
	GG	GA	AA	G	A	
Exposed	17 (9.4%)	31 (17.2%)	132 (73.3%)	0.13	0.87	0.002[a]
Unexposed	3 (1.7%)	23 (12.8%)	154 (85.6%)			
rs1625579 (MIR-137)						
	TT	TG	GG	T	G	
Exposed	120 (66.7%)	55 (30.6%)	5 (2.8%)	0.80	0.20	0.15[a]
Unexposed	108 (60%)	60 (33.3%)	12 (6.7%)			

[a] p value associated to chi-square test.

Indeed, comparing genotoxic damage in individuals who reported using full PPE relative to those who did not use PPE or used incomplete PPE demonstrated higher levels of DNA damage among the latter group ($p < 0.001$). We also found increased DNA damage in older farmworkers and in those that reported intoxication. Such findings demonstrated an influence of PPE's effectiveness in preventing the genotoxic effects of pesticides on peripheral blood cells (Simoniello et al., 2008). Previous studies have shown the same correlations between PPE use, intoxication, age and gender on level of DNA damage and suggested to be due to differences in exposure conditions, DNA repair capability and lifestyle factors (Simoniello et al., 2008; How et al., 2015; Ali et al., 2018; Cayir et al., 2019). According to Ali et al. (2018), women from Bahawalpur District, of the Punjab province, in India, exposed to pesticides while picking cotton with bare hands, presented increased DNA damage when compared to controls. Besides, the DNA damage was positively correlated to age and exposure time, demonstrating that DNA repair capability could be committed.

On the other hand, our results did not show any influence of smoking habits and alcohol consumption on genotoxic damage. Therefore, increased DNA damage in the exposed group was due to exposure to pesticides, and not associated with other confounding factors. Similar results were also produced by other authors, who found no significant difference between lifestyle and DNA damage (Simoniello et al., 2008; Kaur et al., 2011; Wilhelm et al., 2015), unlike Barrón-Cuenca et al. (2019) and Hayat et al. (2019), which showed an increase of DNA damage.

Moreover, $CD4^+$ lymphocytes subpopulations were also analyzed as a biomarker of alterations on the immune system. Our analysis demonstrated significant modifications of immunotoxicity parameters in the exposed group, specifically in the percentages of $TCD4^+$, T $CD4^+CD25^+$ and $TCD4^+CD25^-FOXP3^+$ lymphocytes subsets that indicates that the pesticides evoke an alteration in the $CD4^+$ lymphocytes in the peripheral blood of exposed individuals. However, the percentage of natural regulatory T cells $CD3^+CD4^+CD25^+FOXP3^+$ did not differ significantly between the exposed and the non-exposed groups. Our findings are promising as demonstrated a disturbance in the pattern of $TCD4^+$ lymphocytes subpopulation in front of the pesticide and a maintenance of the natural regulatory T cells percentage. This data strongly suggests that the pesticides evoke a peripheral T cell subpopulation alteration, what is plausible to purpose, would commit further immune reactions. At this moment our group is working to better explore this data.

Another important issue is individual susceptibility that influences physiological responses to pesticide exposure. Therefore, it is essential to identify genotypes that determine the modulation of the proteins involved in the metabolization, detoxification, and DNA repair, influencing the heterogeneity of responses to pesticides (Oliveira et al., 2019, 2019). Regarding susceptibility biomarkers, only TNF-α rs361525 polymorphism showed a significant difference in genotype distributions between exposed and control groups ($p = 0.002$). TNF-α gene encodes tumor necrosis factor-alpha (TNF-α) proinflammatory cytokine that is enhanced by the oxidative stress pesticide-induced as reported by some authors (Mecdad et al., 2011; Gangemi et al., 2016).

In this study, subjects exposed to a complex mixture of pesticides had a significantly positive correlation between the TNF-α (rs361525) polymorphism and the DNA damage ($r = 0.19$; $p = 0.01$). All together, these findings indicate that the higher prevalence of A allele constitutes a susceptibility factor for the DNA damage observed in the pesticide-exposed farmworkers. Finally, genotype and allele frequencies of PON1, XRCC1, IL6, IL6R and MIR-137 were similar to other studies (Wong et al., 2008; Singh et al., 2011; Satti et al., 2013; Mahmoudi and Cairns, 2017) and did not present distinct distribution between exposed and unexposed groups.

5. Conclusions

Overall, the responses to a complex mixture of pesticides exposure vary within and between populations, which indicates the potential influences of genetic, environmental, and lifestyle factors. Our results suggested essential changes in the group of farmworkers, especially concerning DNA damage, reinforcing the fact that the non-use of PPE, intoxication reports, gender and age could increase the extent of DNA damage. Besides, immune changes, such as an increase in the number of lymphocytes, show an immune activation in defense against xenobiotics. Further studies are needed and should apply biomarkers to detect early effects of pesticide exposure and prevent chronic outcomes. Therefore, based on our results, the studied biomarkers are useful in assessing the occupational and environmental exposure to pesticides and estimating risk for long-term deleterious health effects. Finally, we suggested a general overhaul of the Brazilian public policies regulating pesticide certification and commercialization to reduce exposure, risks, and the negative consequences for human and environmental health. Also, enlightenment programs on safety precautions are crucial to increase the rural population awareness of the risks from pesticide exposure.

CRediT authorship contribution statement

Jheneffer Sonara Aguiar Ramos: Conceptualization, Methodology, Validation, Writing - original draft, Visualization, Project administration. **Thays Millena Alves Pedroso:** Methodology, Validation, Visualization. **Fernanda Ribeiro Godoy:** Conceptualization, Methodology, Validation, Project administration. **Renata Elisa Batista:** Methodology. **Frankcione Borges de Almeida:** Methodology. **Carolina Francelin:** Methodology, Validation, Writing - original draft. **Francis Lee Ribeiro:** Methodology. **Michelle Rocha Parise:** Methodology, Validation, Resources, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition. **Daniela de Melo e Silva:** Conceptualization, Methodology,

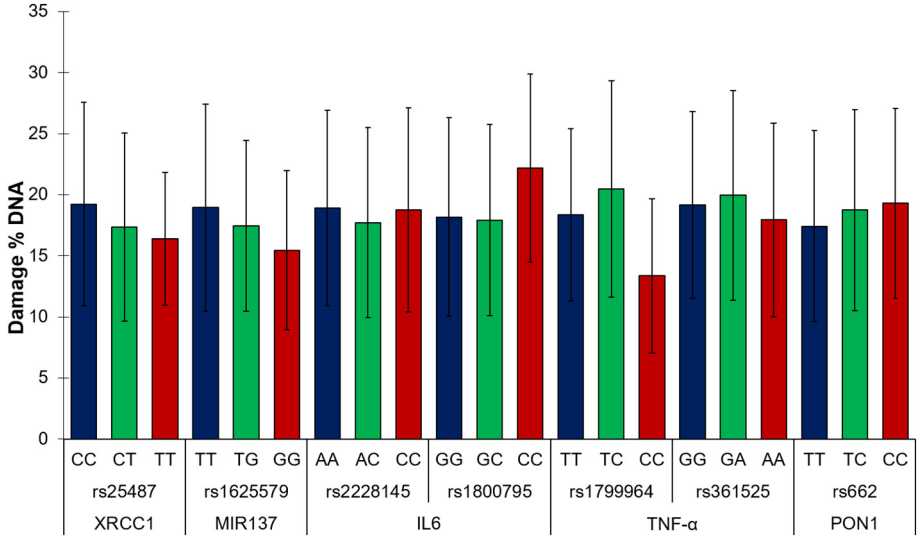

Fig. 4. Association between genotypes frequencies of XRCC, MIR137, IL6, TNF-α and PON1 genes and % DNA in tail (% DNA).

Resources, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition.

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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