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Relationship between PON-1 enzymatic activity and risk factors for pesticide poisoning in farmers from the Cienega, Jalisco, Mexico

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Abstract: Paraoxonase-1 (PON-1) is an enzyme that hydrolyzes organophosphate pesticides. The presence of polymorphisms in PON-1 (L55M and Q192R) decreases its enzyme activity and increases the risk of central nervous system (CNS) toxicity in occupationally exposed farmers, leading to chronic degenerative diseases and death. We studied 103 farmers in the region of Cienega Jalisco, Mexico, which were exposed mainly to organophosphate pesticides. We used serum and plasma samples to assay PON-1 activity and perform polymorphism analysis (L55M and Q192R) using qPCR and TaqMan probes, respectively. For both polymorphisms, there was high percentage of heterozygosity (55 LL = 0.19, LM = 0.75, MM = 0.06; 192 QQ = 0.12, QR = 0.72, RR = 0.16), while the allelic frequencies were more balanced (L = 0.56, M = 0.44; Q = 0.48, R = 0.52). There were no significant differences in enzyme activity of L55M polymorphism genotypes (LL = 179.27; LM = 192.11; MM = 122.11; QQ = 135.74; QR = 187.90; RR = 209; $p > 0.05$). But there was a slight decrease in enzyme activity for the Q192R polymorphism genotypes. The genotype and alcohol consumption associated with slight increases in enzyme activity. However, genotype and tobacco consumption did not have a significant effect on PON-1 activity (μU/mL) ($p > 0.05$). Overall, alcohol and tobacco consumption affected PON-1 enzyme activity (μU/mL) up to 21.1%. The data obtained in this study reveal that PON-1 activity is affected by genetic variants such as Q192R and alcohol consumption. This may influence the susceptibility of populations to organophosphate poisoning.

Introduction

Pesticides are chemicals that prevent or destroy pests and are used primarily in agriculture to increase crop production and profitability; however, they are highly toxic to non-target organisms. Previous evidence indicates that exposure to pesticides decreases the enzymatic activity of enzymes such as carbonic anhydrase and glutathione-S-transferase. (Caglayan *et al.*, 2019; Özaslan *et al.*, 2018; Amr *et al.*, 2015; Costa *et al.*, 2015; Lozano-Paniagua *et al.*, 2016; Muñoz-Quezada *et al.*, 2016). Approximately 4 million tons/year are applied worldwide, out of which only 1% is estimated to have direct contact with pests (Amr *et al.*, 2015). Pesticide toxicity in humans is mainly due to inappropriate use and mismanagement, as well as acute or chronic exposure, in addition to its multiple routes of human internalization such as a dermal route, inhalation, and ingestion (Lozano-Paniagua *et al.*, 2016; Muñoz-Quezada *et al.*, 2016).

According to the World Health Organization (WHO), about 3 million cases of intoxication and 220,000 deaths occur each year due to pesticides (Ali and Chia, 2008; Marsillach *et al.*, 2016). The most widely used pesticides in the world are organophosphates (OPs), compounds derived from phosphoric acid esters. In humans, these compounds are activated via oxidation by the cytochrome P450 enzyme to ozons, toxic metabolites which are inhibitors of the enzyme acetylcholinesterase (AChE) (Dardiotis *et al.*, 2019; Paul *et al.*, 2017). Acetylcholine esterase catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh). Thus, the inhibition of AChE produces an increase in the level of the neurotransmitter substrate (ACh), causing a malfunction in the cholinergic synapses of the central nervous system (CNS) by increasing the transmission of electrical impulses (Gündoğdu *et al.*, 2019). This produces what is known as a pesticide-induced cholinergic syndrome, which is characterized by symptoms such as excessive

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secretions from excretory glands, loss of consciousness, and respiratory and cardiac problems, which can cause death (Ali and Chia, 2008; Dardiotis *et al.*, 2019; Sirivarasai *et al.*, 2007; Durgun *et al.*, 2020; Sever *et al.*, 2020).

Paraoxonase-1 (PON-1) is an enzyme that hydrolyzes OP-type compounds. The first known substrate for the enzyme was paraoxon, a toxic metabolite of parathion, hence its name.

PON-1 hydrolyzes paraoxon to p-nitrophenol, but it also shows a high affinity for other molecules with free amino and hydroxyl groups (Demir *et al.*, 2019; Demir, 2020). PON-1 is a multifunctional enzyme with the capacity to act as arylesterase, lactonase, and organophosphatase (Xotlanihua-Gervacio *et al.*, 2019; Mitra *et al.*, 2015). PON-1 is a 43-kDa calcium-dependent glycoprotein expressed by the 9-exon PON-1 gene located on the long arm of chromosome 7 (7q21); it is synthesized in the liver and secreted into the bloodstream via association with high-density lipoproteins (HDL) (Xotlanihua-Gervacio *et al.*, 2019; López-Flores *et al.*, 2009; Mitra *et al.*, 2015; Mota *et al.*, 2019; Demir, 2019). The binding of PON-1 to HDL depends on terminal amino residues that anchor to phospholipids. PON-1 has two critical calcium ions, one has a structural function, and the other is necessary for its catalytic activity. The active site of PON-1contains tryptophan, histidine, lysine, phenylalanine, and aspartate/glutamine residues. At the active site, calcium ion interacts with Asn 224, 270, 168, Asp 269, and Glu 53 residues. (Demir and Beydemir, 2015). Two polymorphisms in the coding region affect the activity of the enzyme: one at position L55M and the other at position Q192R. Polymorphism 192 (rs662 A > G) causes a change from glutamine (Q) to arginine (R) that affects the catalytic efficiency of the enzyme towards different substrates. For example, alloenzyme Q hydrolyzes diazoxon more effectively than alloenzyme R; whereas, alloenzyme R hydrolyzes paraoxon more effectively than alloenzyme Q. Polymorphism 55 (rs854560 A > T) has a substitution of leucine (L) by methionine (M), which is associated with a low serum concentration of the enzyme. This effect could be due to the low levels of PON-mRNA1, as a result of regulation by the ligation of the M variant with 108 C/T promoter region polymorphism (Costa *et al.*, 2003; López-Flores *et al.*, 2009; Pan *et al.*, 2019; Sirivarasai *et al.*, 2007). The objective of this study was to investigate the relationship between the enzymatic activity of PON-1 and its genetic polymorphisms (L55M and Q192R) and other variables, such as alcohol and tobacco consumption, in farmers exposed to pesticides from the region of the Cienega Jalisco, Mexico.

Materials and Methods

Study population

We collected 103 blood samples from farmers with occupational exposure to different pesticides (Tab. 1) from 14 localities in 3 municipalities in the region of the Cienega Jalisco Mexico. The exclusion criteria included subjects younger than 18 and older than 60 years of age, subjects with kidney and liver diseases, diabetes, hypertension, cardiovascular diseases, and cancer, on lipid-lowering treatment or illegal drug use. We were sampling during the months of April and May, which includes a period of intensive agriculture activity that involves preparations for the planting of corn in this region. We collected blood samples into sample tubes with or without EDTA (for plasma or serum samples, respectively). We used plasma and serum fractions for genotyping analysis and enzyme activity assays, respectively. In addition, we applied a survey to each farmer to identify the type of pesticide to which they are exposed.

PON-1 genotyping

In this study, DNA was extracted from the samples using Quick-DNATM Miniprep kit Catalog Nos. D3024 and D3025 of ZYMO RESEARCH CORP, in line with the manufacturer's instructions. The PON-1 genotypes for

TABLE 1

Pesticides used by farmers and their classification according to Environmental Protection Agencies and the World Health Organization

Bioactive principle	Chemical family	US EPA	WHO
Diazinon	Organophosphates	E	II
Parathion		C	IA
Terbufos		E	IA
Chlorpyrifos		C	II
Malathion		D	II
Carbofuran	Carbamate	NL	1B
Cyhalothrin	Pyrethroid	D	II
Cypermethrin		C	II
Deltamethrin		NL	II
Paraquat	Bipyridyl	E	II
Atrazine	Triazine	NL	III

Note: U.S. EPA (Environmental Protection Agency)-Carcinogenicity Categorization: B2, Probable human carcinogen; C, Possible human carcinogen; D, Not classifiable as to human carcinogenicity; E, Evidence of not carcinogenicity for humans. NL, Not likely to be carcinogenic to humans. WHO (World Health Organization)-classification of pesticides by hazard: IA, Extremely hazardous; IIB, Highly hazardous; II, Moderately hazardous; III, Slightly hazardous; U, Unlikely to present acute hazardous effect.

polymorphisms 55 and 192 were determined with real-time PCR using Taqman® assays SNP Genotyping kit (ThermoFisher-Scientific), with identification number per test C_ _ _2259750_20 y C_ _ _ 2548962_20, respectively. Each amplification reaction mixture comprised 5 µl of TaqMan Universal PCR Master Mix from Applied Biosystems (containing AmpliTaq Gold DNA Polymerase UP, dNTPs with dUTP, ROX Passive Reference and optimized buffer components); 0.5 µL of Taqman® assay SNP Genotyping (containing wild and mutated TaqMan probe, and primers: forward and reverse); 0.5 µL genomic DNA containing approximately 20 ng, and 10 µL of DNase-free water. The reaction was amplified in a thermocycler LightCycler® Nano (Roche) under the following conditions: a preheating stage of 50°C per 2 min, a heating stage at 95°C for 10 min, and an amplification stage at 45 cycles of 95°C at 15 s, and 60°C for 1 min. At the end of the real-time PCR, the genotypes were analyzed with endpoint genotyping analysis that measured the distribution of fluorescence intensity of the TaqMan probe tags for detection of wild-type and mutated sequences (probe fluorophores: 6-FAM and VIC).

Enzymatic activity of PON-1

The activity of PON-1 in serum was measured using Paraoxonase-1 Activity Assay Kit (BioVision Incorporated) and paraoxon as substrate. The principle of the assay consists of the spectrofluorometric measurement (Ex/Em = 368/460 nm) of p-nitrophenol, which is formed as a yellow chromogenic product by the hydrolytic activity of PON-1. The reactions were performed in a volume of 80 µL, adding 5 µL of serum per sample. The plate was pre-incubated for 10 min at 37°C, and subjected to the kinetic mode for 10 min, taking readings every minute at 37°C. The test contained a positive control with PON-1. The negative control contained a 2-hydroxyquinoline inhibitor. Two time points (t1 and t2) in the linear phase of the progress curves of each reaction were used to determine PON-1 activity, obtaining the corresponding fluorescence values at those points (RFU1 and RFU2) to determine the change in fluorescence over time: FS = RFU2 − RFU1. The specific fluorescence (CS) was calculated by subtracting the control background (FBC) of each sample: CS = FS − FBC. The CS values were obtained from the standard fluorescence curve to obtain B pmol of the metabolized substrate during the reaction time and the enzymatic activity of PON-1 was calculated by the following formula:

$$\text{PON1 Activity (A)} = \frac{\text{B}}{(\Delta\text{T} * \text{V})} * \text{D}$$

- A = paraoxonase activity (pmol/min/mL = µU/mL).
- B = amount of metabolite produced calculated from the standard curve (pmol).
- ΔT = linear phase reaction time t2 − t1 (min).
- V = sample volume added in the sampling wells (mL).
- D = dilution factor of the sample if diluted to conform to the standard curve range (before preparation of the reaction well).

Statistical analysis

Appropriate statistical treatment of the data is essential. Allelic and genotypic frequencies, observed and expected heterozygosity, ligation imbalance, inbreeding coefficient (*Fis*), and Hardy–Weinberg equilibrium were determined through the Genetic Data Analysis (GDA) program (Lewis and Zaykin, 2001). The levels of significance were determined in 10000 simulations. Subjects were classified according to their genotypes (PON-1 55; LL, LM, MM and PON-1 192; QQ, QR and RR). The genotypes were tested for their association with enzymatic activity and various factors such as exposure to pesticides OPs, age groups (<20, 21–30, 31–40, 41–50, and >50 years), consumption of alcohol and tobacco. The comparison of enzymatic activity according to genotype was performed using ANOVA and Kruskal-Wallis tests. We also compared the mean values for the groups using the Student's *t*-test and Mann Whitney's U-test. Finally, the association between genotypes and different variables was determined using single and multiple linear regression analyses. All tests were performed using the statistical program IBM SPSS v.25.0. Values of $p < 0.05$ were indicative of statistical significance.

Results

Pesticides used in the region of the Cienega Jalisco, Mexico

The participants indicated that they used 7 different classifications of pesticides, being organophosphate type the most used (59.64%), followed by pyrethroids (12.93%), carbamates (11.11%), and bipyridyls (9.75%). These results are shown in Tab. 2. The organophosphate pesticides most used were terbufos and chlorpyrifos (21.5% and 18.6%, respectively), while the most frequently used pesticide with herbicide function was glyphosate (16.1%).

Characteristics of the study population

The study was carried out on farmers mainly exposed to OPs. The participants were mostly men (96.1%), with a mean age of 41 years. Approximately 84% of the participants had more than 3 years of pesticide exposure, while 33% of the farmers had experienced some form of pesticide poisoning. Tab. 3 shows the detailed characteristics of the study population, including other important parameters that directly affect the enzymatic activity of PON-1, such as alcohol consumption (78.3%) and tobacco use (26.1%).

PON-1 genotypes and allelic frequencies

The allelic and genotypic frequencies of the PON1 coding region polymorphisms are shown in Tab. 4. We observed a high frequency of heterozygous genotypes for each polymorphism. The Hardy–Weinberg equilibrium was calculated, and statistical significance was obtained ($p < 0.05$). Therefore, the results suggest that the population was not in equilibrium. In addition,

the calculated ligation imbalance was statistically significant ($p < 0.05$). The value of the inbreeding coefficient *Fis* was −0.482345.

Enzymatic activity of PON-1 as a function of its genotypes

We classify the enzymatic activity of PON-1 according to its genotype (Tab. 5) and made comparisons between the L55M polymorphism and its genotypes, and the result did

TABLE 2

The most used pesticides in the study population

Type	Chemical family (%)	Bioactive principle	Frequency of use (%)
Insecticide	Organophosphates (59.64)	Diazinon	7 (1.6)
		Parathion	3 (0.7)
		Methamidophos	1 (0.2)
		Terbufos	95 (21.5)
		Chlorpyrifos	82 (18.6)
		Acephate	1 (0.2)
		Malathion	3 (0.7)
	Carbamate (11.11)	Carbofuran	48 (10.9)
		Methomyl	1 (0.2)
	Pyrethroid (12.93)	Cyhalothrin	29 (6.6)
		Cypermethrin	22 (5.0)
		Deltamethrin	3 (0.7)
		Bifenthrin	2 (0.5)
		Cyfluthrin	1 (0.2)
	Abamectin (1.59)	Emamectin	2 (0.5)
		Abamectin	5 (1.1)
Herbicide	Organophosphate (59.64)	Glyphosate	71 (16.1)
	Bipyridyl (9.75)	Paraquat	43 (9.8)
	Sulfonylurea amine (4.08)	Nicosulfuron	18 (4.1)
	Triazine (0.91)	Atrazine	4 (0.9)

TABLE 3

Socio-demographic characteristics of the study population

Characteristic	*
Age (years)	41.03 ± 18.34
Weight (kg)	90.69 ± 67.38
Size (cm)	173.60 ± 8.68
Characteristics	N (%)
Sex	
F	4 (3.9)
M	99 (96.1)
Municipalities	
La Barca	41 (41.0)
Atotonilco el Alto	32 (32.0)
Poncitlan	27 (27.0)
Alcohol consumption	
Yes	54 (78.3)
Not	15 (21.7)
Tobacco consumption	
Yes	18 (26.1)
Not	51 (73.9)
Pesticide exposure (years)	
Acute (< 3)	16 (15.8)
Intermediate (3–10)	24 (23.8)
Chronic (> 10)	61 (60.4)
Subjects with pesticide poisoning	
Yes	34 (33.3)
No	68 (66.7)

Note: *Values are expressed as mean ± SD, or N (%).

not show significant differences ($p > 0.05$). However, we observed that the mutated homozygous genotype had lower activity. Also, we did not find statistical significance ($p > 0.05$) between the Q192R polymorphism and its genotypes, but the wild-type homozygous genotype had lower enzymatic activity relative to the mutated genotype.

Enzymatic activity of PON-1 and non-genetic parameters

The enzymatic activity of PON-1 was compared with respect to the genotype of each polymorphism, alcohol consumption, tobacco use, age groups (<20, 21–30, 31–40, 41–50, and 50 years), and type of exposure (acute, intermediate, and chronic). For these comparisons, we found statistical significance only with alcohol consumption ($p = 0.011$) in relation to the LM genotype of the L55M polymorphism (Tab. 6). We also found statistical significance for the QR and RR genotypes of the Q192R polymorphism ($p = 0.000$ and $p = 0.032$, respectively) (Tab. 6). We also compared the enzyme activity as a function of genotype in tobacco

TABLE 4

PON-1—Allelic and genotypic frequencies

	Polymorphisms			
	55 (L/M)	**N**	**192 (Q/R)**	**N**
Genotype	LL (0.19)	18	QQ (0.12)	12
	LM (0.75)	73	QR (0.72)	70
	MM (0.06)	6	RR (0.16)	15
Allele	L (0.56)		Q (0.48)	
	M (0.44)		R (0.52)	
P [a]	$P < 0.05$		$p < 0.05$	
P [b]	$P < 0.05$			
Fis	−0.482345			

Note: a Hardy–Weinberg equilibrium; b LD: Linkage disequilibrium; *Fis*: Inbreeding coefficient.

TABLE 5

PON-1 activity according to genotype

Polymorphisms	**N**	**PON-1 (μU/mL)** $\bar{X} \pm \sigma$	***P*** [a b]	***P*** [c d]
55				
LL	18	179.27 ± 99.64	LL *vs.* LM: $P^a = 0.702$	$P^c = 0.416$
LM	73	192.11 ± 119.41	LL *vs.* MM: $P^a = 0.267$	
MM	6	122.11 ± 85.46	LM *vs.* MM: $P^a = 0.206$	
192				
QQ	12	135.74 ± 113.82	QQ *vs.* QR: $P^b = 0.224$	$P^d = 0.327$
QR	70	187.90 ± 119.26	QQ *vs.* RR: $P^b = 0.100$	
RR	15	209.51 ± 82.37	QR *vs.* RR: $P^a = 0.552$	

Note: a Student's *t*-test; b Mann–Whitney U-test; c ANOVA; d Kruskal-Wallis test.

TABLE 6

PON-1 activity as a function of genotype and alcohol consumption

Polymorphism		**Alcohol consumption PON-1 (μU/mL)**		***P*** [a b]
	N	**YES** ($\bar{X} \pm \sigma$)	**NO** ($\bar{X} \pm \sigma$)	
55				
LL	18	147.01 ± 112.72	90.21 ± 41.19	$P^a = 0.433$
LM	73	210.80 ± 119.77	101.84 ± 98.66	$P^a = 0.011$
MM	6	149.58 ± 104.74	109.29	NA
192				
QQ	12	76.57 ± 89.68	246.01	NA
QR	70	209.63 ± 113.11	50.10 ± 34.66	$P^a = 0.000$
RR	15	279.02 ± 62.98	169.24 ± 69.98	$P^a = 0.032$

Note: a t-Student; NA: Not applicable.

consumers (Tab. 7), and in both polymorphisms, we did not find statistical significance ($p > 0.05$). However, we observed a reduction in enzyme activity in most genotypes.

We used linear single and multiple linear regression models to determine the association between genotype, alcohol and tobacco consumption, and PON-1 activity. The multiple regression model shown an R_2 value of 0.211, which indicated that the genotypes for both polymorphisms (alcohol and tobacco consumption) affected the enzymatic activity of PON-1; in contrast, the single linear regression model indicated a poor relationship (Tab. 8).

Discussion

Pesticides used in the region of the Cienega Jalisco, Mexico

Most of the participants in this study were males (96.1%). This could be an advantage since there is less variability in the enzymatic activity of PON-1 with respect to the female sex (Fridman *et al.*, 2001). Moreover, the ages of the participants ranged between 18 and 60 years, with a mean age of 41 years, which coincides with the age of greatest occupational exposure to pesticides in farmers. This is in agreement with a previous report in Mexico where the age range was 18 to 58 ± 5 years at the upper limit (López-Flores *et al.*, 2009; Pérez-Herrera *et al.*, 2008; Rojas-García *et al.*, 2005). Sixty percent (60%) of participants were farmers with chronic occupational exposure (>10 years). The frequency of exposure to OPs was higher (approximately 60%) than that of other pesticides. This may be related to CNS toxicity, immunosuppression, genotoxicity and cancer (Androutsopoulos *et al.*, 2011; Arévalo-Jaramillo *et al.*, 2019; Ceja-Gálvez *et al.*, 2020; Martínez-Valenzuela and Gómez-Arroyo, 2007; Reynoso *et al.*, 2019; Suratman *et al.*, 2015). The frequent use of OPs is due to their effectiveness in the control of corn pests (Murcia and Stashenko, 2008). The region of the Cienega is a major producer of this grain. Indeed, this region uses OPs more frequently than florists in the state of Mexico and Morelos, with a frequency of use of 49% (López-Flores *et al.*, 2009). Thus, they are more susceptible to the health risks associated with these pesticides. Terbufos and chlorpyrifos are the most used. A study carried out in the region of Coquimbo, Chile, revealed that the most widely used pesticide was chlorpyrifos (Zúñiga-Venegas *et al.*, 2015). The use of this pesticide may be responsible for the greater susceptibility to poisoning in farmers with the wild-type QQ genotype of the 192 polymorphism since the QQ alloenzyme presents a low capacity for metabolizing this substrate (Ceja-Gálvez *et al.*, 2020; Torres-Sánchez *et al.*, 2019). Regarding the use of herbicides, the Cienega in Jalisco and the states of Mexico and Morelos use mainly glyphosate, which is interesting, since the International

TABLE 7

PON-1 activity as a function of genotype and tobacco consumption

Polymorphisms		Tobacco use PON-1 (μU/mL)		$P^{a\ b}$
	N	YES ($\bar{X}$ ± σ)	NO ($\bar{X}$ ± σ)	
55				
LL	18	140.93 ± 105.18	75.94	NA
LM	73	185.17 ± 127.12	202.17 ± 106.49	$P^a = 0.699$
MM	6	30.66	175.79 ± 60.68	NA
192				
QQ	12	69.23 ± 117.88	142.85 ± 83.20	$P^b = 0.289$
QR	70	179.08 ± 127.53	176.26 ± 86.32	$P^a = 0.95$
RR	15	215.59 ± 78.85	330.25	NA

Note: a Student's *t*-test; b Mann–Whitney U-test; NA: Not applicable.

TABLE 8

Linear single and multiple linear regression analyses for PON-1

Variable	Simple			Multiple		
	Coefficient β	*p*	R^2	Coefficient β	*p*	Total R^2
L55M Polymorphism	0.07	0.63	−0.016	0.162	0.238	
Q192R Polymorphism	0.16	0.16	0.013	0.411	0.004	
Alcohol	−0.318	0.02	0.084	−0.414	0.003	
Tobacco	0.03	0.981	−0.02	0.001	0.992	0.211

Agency for Research on Cancer (IARC) decreed in 2015 that glyphosate is a potent carcinogen (Reynoso *et al.*, 2019). A previous study suggested that glyphosate may be nephrotoxic when it interacts with metal compounds in hard water, which could be responsible for epidemics of chronic kidney disease (Jayasumana *et al.*, 2014).

PON-1 genotypes and allelic frequencies

We found a high percentage of heterozygous genotypes for L55M polymorphism (0.75). This differs from the results of related studies in Mexico (Gamboa *et al.*, 2006; López-Flores *et al.*, 2009; Pérez-Herrera *et al.*, 2008; Rojas-García *et al.*, 2005; Torres-Sánchez *et al.*, 2019) that showed heterozygosity >30% with the most common wild-type homozygous genotypes. However, it is possible that the heterozygosity of this polymorphism may be influenced by the genetic linkage it showed with the Q192R polymorphism. Allelic frequency was more balanced, but the wild-type allele was slightly more frequent (0.56). It is worth mentioning that in other studies carried out in Mexico, the wild-type allele presented a higher frequency, compared with the region of the Cienega (Gamboa *et al.*, 2006; López-Flores *et al.*, 2009; Pérez-Herrera *et al.*, 2008; Rojas-García *et al.*, 2005; Torres-Sánchez *et al.*, 2019). We found a high percentage of heterozygous genotypes for Q192R (0.72). This is consistent with the results of studies carried out in Mexico and Peru. The Peruvian study showed more comparable results to the region of Cienega, with respect to genotypic frequency (0.61) (Cataño *et al.*, 2006). Regarding allelic frequencies, we found a higher percentage of the mutated allele (0.52). This result is similar to that reported in a study carried out on farmers from Yucatan, Mexico, whose mutated allele had a frequency of 0.53 (Pérez-Herrera *et al.*, 2008). It is worth mentioning that there are other studies that report different but relatively close allelic frequencies (Cataño *et al.*, 2006; Gamboa *et al.*, 2006; López-Flores *et al.*, 2009; Pérez-Herrera *et al.*, 2008; Rojas-García *et al.*, 2005; Torres-Sánchez *et al.*, 2019; Zúñiga-Venegas *et al.*, 2015).

Regarding the heterozygotes of both polymorphisms, there was no Hardy–Weinberg equilibrium. Thus, the inbreeding coefficient *Fis* was calculated, resulting in a value of −0.482345, indicating that for both polymorphisms, there was an excess of 48% heterozygotes. Knowing that the high percentage of heterozygous may be due to technical genotyping problems, the procedure was verified in an external laboratory by sequencing selected samples for each genotype. This confirmed our results. However, it should be mentioned that there are other evolutionary factors such as gene flow, natural selection, and inbreeding, which can also influence allelic distribution (Mohammadi *et al.*, 2015). The ligation imbalance was also calculated, obtaining a significant value ($p < 0.05$), which could explain the high percentage of heterozygotes.

Enzymatic activity of PON-1 as a function of its genotypes

The L55M polymorphism is known to result in reduced enzyme activity because there is a lower expression of the enzyme and therefore a lower concentration in the circulatory system. However, analysis of the relationship between genotypes and enzymatic activity did not show a significant trend ($p > 0.05$). In contrast, some trends were reported in other studies in which mutated homozygotes presented less PON-1 activity than wild-type homozygotes (Gamboa *et al.*, 2006; López-Flores *et al.*, 2009; Pérez-Herrera *et al.*, 2008; Rojas-García *et al.*, 2005; Torres-Sánchez *et al.*, 2019). The Q192R polymorphism causes a switch from glutamine to arginine. This causes a conformational change in the active site of PON-1, resulting in changes in the enzyme activity. In addition, the activity of the enzyme is influenced by differences in the affinities towards different substrates to be metabolized. In our study, we did not find significant differences that indicate that the genotypes of this polymorphism affected enzymatic activity. In contrast, other studies carried out in Mexico, Chile and Peru have reported that mutated homozygotes had greater activity than wild-type ones, when using paraoxon as substrate (Cataño *et al.*, 2006; Gamboa *et al.*, 2006; López-Flores *et al.*, 2009; Pérez-Herrera *et al.*, 2008; Rojas-García *et al.*, 2005; Torres-Sánchez *et al.*, 2019; Zúñiga-Venegas *et al.*, 2015).

Relationship between PON-1 activity and non-genetic parameters

In addition to genetic polymorphisms, the activity of PON-1 is affected by different variables such as diet, alcohol and tobacco consumption, environmental toxins, aging, pregnancy, and various pathologies (Ceja-Gálvez *et al.*, 2020). We found that alcohol consumption affected PON-1 activity ($p < 0.05$), with respect to the heterozygotes of both polymorphisms and the mutated homozygous of the Q192R polymorphism, in which we observed increases in PON-1 activity. This is consistent with previous research where moderate alcohol consumption slightly increased PON-1 activity, while excessive alcohol exposure led to decreased enzyme activity (Gruppen *et al.*, 2018; Rao *et al.*, 2003; Xotlanihua-Gervacio *et al.*, 2019). Similarly, a study in rats showed that light alcohol consumption regulated the expression of PON-1 mRNA (Rao *et al.*, 2003). Additionally, a human study reported that the threshold for alcohol consumption to increase PON-1 activity ranged from 10 to 30 g/day (Gruppen *et al.*, 2018). Comparisons between tobacco use and any of the genotypes of both polymorphisms did not show any statistically significant effect on enzyme activity ($p > 0.05$). However, we observed trends in which the genotype of tobacco users resulted in slightly reduced PON-1 activity. This may be due to oxidative stress produced by cigarette smoke which contains several oxidizing and pro-oxidant substances capable of producing reactive oxygen species with hydroxyl radicals, resulting in the inactivation or oxidization of PON-1, thus decreasing its activity (Bizoń *et al.*, 2016; Kahraman *et al.*, 2017; Marek *et al.*, 2018; Xotlanihua-Gervacio *et al.*, 2019). At the same time, it should be noted that there are studies that have shown that cigarette smoking affects the activity of the PON-1 enzyme by up to 40% (Bizoń *et al.*, 2016).

The effect of tobacco and alcohol consumption on PON-1 enzyme activity and genetic polymorphism

PON-1 enzyme activity can vary in response to external and genetic factors, so we performed a single linear regression to

find out if each of the variables (polymorphisms, alcohol, and tobacco consumption) affected the enzyme. The results showed a poor association between enzyme activity and these variables; only the Q192R polymorphism and alcohol consumption showed low correlation values (1.3% and 8.4%, respectively). However, multiple linear regression analysis showed that these variables affected PON-1 activity by up to 21.1%. A study carried out in Spain showed that polymorphisms (L55M and Q192R), alcohol consumption, tobacco use, gender, physical activity, cholesterol, triglycerides, HDL, body mass index, and nutrition (consumption of antioxidants and lipids) can influence 74% of PON-1 activity (Ferrè *et al*., 2003). In another study, in Mexico, it has been observed that four polymorphisms (−162, −108, L55M, and Q192R) caused a change in PON-1 of up to 25% (Rojas-García *et al*., 2005). This is a similar 21.1% change observed in this study when two polymorphisms (L55M and Q192R) were tested in relation to factors such as alcohol and tobacco consumption. Finally, our study shows that the additive effect of genetic variants of PON-1 and the consumption of alcohol and tobacco allow an association of susceptibility or risk of poisoning due to occupational exposure to pesticides. However, cohort studies are needed to assess whether genetic variants and other factors can improve the prediction of poisoning among farmers.

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Ethics Approval: The Ethics Committee of the University Center of the Cienega, University of Guadalajara, approved the experimental protocol carried out in this project (Folio 2017–037). Each participant signed an informed consent letter guaranteeing the confidentiality of the data. The study was conducted in strict compliance with the principles of the Helsinki Declaration.

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