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Polymorphisms of pesticide-metabolizing genes in children living in intensive farming communities

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HIGHLIGHTS

- 10 polymorphic gene variants of pesticide-metabolizing enzymes were analyzed.
- Allelic frequency, linkage disequilibrium and haplotype analysis were studied.
- SNPs and CNVs frequencies were broadly consistent with European populations.
- Adverse genotype combinations conferring a greater genetic risk were suggested.
- These combinations can be used in future studies to predict adverse health effects.

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ABSTRACT

Polymorphisms in genes encoding xenobiotic-metabolizing enzymes (XME) are important parameters accounting for the wide inter-individual variability to environmental exposures. Paraoxonase-1 (PON1), butyrylcholinesterase (BChE) and Cytochrome-P450 constitute major classes of XME involved in the detoxification of pesticide chemicals, in particular organophosphates. This study explored the allelic frequency, linkage disequilibrium and haplotype analysis of ten common polymorphic variants of seven key genes involved in organophosphate metabolism (*BCHE-K*, *BCHE-A*, *PON1 Q192R*, *PON1 L55M*, *PON1 −108C/T*, *CYP2C19 G681A*, *CYP2D6 G1846A*, *CYP3AP1 −44G/A*, *GSTM1*0* and *GSTT1*0*) in a children population living near an intensive agriculture area in Spain. It was hypothesized that individuals with unfavorable combinations of gene variants will be more susceptible to adverse effects from organophosphate exposure. Genomic DNA from 496 healthy children was isolated and amplified by PCR. Hydrolysis probes were used for the detection of eight specific SNPs and two copy number variants (CNVs) by using TaqMan® Assay-based real-time PCR. Frequencies of SNPs and CNVs in the target genes were in Hardy–Weinberg equilibrium and broadly consistent with European populations. Linkage disequilibrium was found between the three *PON1* genetic polymorphisms studied and between *BCHE-K* and *BCHE-A*. The adverse genotype combination (unusual *BCHE* variants, *PON1 55MM/−108TT* and null genotype for both *GSTM1* and *GSTT1*) potentially conferring a greater genetic risk from exposure to organophosphates was observed in 0.2% of our study population. This information allows broadening our knowledge about differential susceptibility toward environmental toxicants and may be helpful for further research to understand the inter-individual toxicokinetic variability in response to organophosphate pesticides exposure.

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1. Introduction

Genetic variations in genes encoding xenobiotic-metabolizing enzymes (XME) largely account for the variability in the response of individuals to environmental chemicals. These variations have been associated with differences in toxicokinetic of xenobiotics and may modify the bioeffective dose at critical target organs, eventually resulting in toxicity from environmental and occupational chemical exposures. Thus, genetic differences in these enzymes may entail an increased risk of (or susceptibility to) environmentally and occupationally related diseases (Tanaka, 1999).

Long-term low-dose exposure to pesticides has been associated with an increased risk of developing a number of chronic diseases, including cancers at different sites, neurodegenerative disorders, neuropsychological deficits and congenital malformations (González-Alzaga et al., 2014; Dardiotis et al., 2013; Mostafalou and Abdollahi, 2013; Parrón et al., 2011, 2014), as has been observed in our study area. However, epidemiological studies have provided conflicting results owing to the late onset of effects and the possible contribution of other causal factors. One potential effect modifier in these studies is genetic variability of pesticide metabolism genes, which at the same level of exposure may allow individuals to metabolize these compounds at different rates (Howard et al., 2010), rendering a distinct metabolite profile. These genetically-based metabolic differences may determine whether or not clinical symptoms (or even intoxication) appear and also predispose individuals to the aforementioned chronic diseases after long-term pesticide exposure.

Although all the pathways for pesticide detoxification are not fully understood, animal studies have indicated the involvement of three main systems in organophosphates (OPs) metabolism: the cytochrome P450 enzymes (CYP2D6, CYP2C19, CYP3A), glutathione S-transferases (GSTM1, GSTT1), and the esterases paraoxonase-1 (PON1), butyrylcholinesterase (BChE) and carboxylesterases (CEs) (Araoud, 2011; Liu et al., 2006). Thus, inheritance of the unfavorable versions of the different polymorphic genes may render an individual more or less susceptible to the adverse effects of pesticides (Bolognesi, 2003) and hence they are strong candidate susceptibility factors for pesticides and diseases. Interestingly, these XME can also metabolize chemicals other than pesticides, such as pharmaceuticals (CYP450s) and reactive species (PON1, GSTs).

Although the individual allelic distribution of the major genes encoding for XME is well established in various populations, information on the degree of linkage disequilibrium between polymorphic variants (single nucleotide polymorphisms –SNPs– and copy number variants –CNVs–) and haplotype distribution is limited. In this study, a healthy Spanish children population living in intensive agriculture areas where pesticides are largely used was screened for genetic polymorphisms in the genes coding for the most important OPs pesticide-metabolizing enzymes (BCHE, PON1, CYP450 and GSTs). In the study area (Southeastern Spain), the amount of insecticides–acaricides–nematocides used accounts for 66.8% of the total pesticide consumption in such area, with OPs representing about 30% of the total amount (tons) of insecticides–acaricides–nematocides sold (Gómez-Martín et al., 2015). The frequencies of SNPs and CNVs as well as the prevalence of haplotypes and combined polymorphisms were explored in order to identify those individuals most at risk for potential adverse health effects from chronic exposure to OPs pesticides.

2. Materials and methods

2.1. Study population

Participant's recruitment took place in public schools from Almeria, Granada and Huelva, three provinces of Andalusia (South Spain) with large areas devoted to intensive agriculture under plastic greenhouses where horticultural crops are grown: 26,264 ha in the province of Almería and 2814 ha in Granada coastline. The coast of the province of Huelva is another target area of plastic greenhouses (1213 ha) where strawberries crops are mostly grown. These areas were considered as of high pesticide use in previous studies (Parrón et al., 2011, 2014). Sixteen schools were randomly selected from the 155 public schools existing in the study area (Adra, Almeria, El Ejido, Berja and Vicar in Almeria province; Almonte, Huelva and Moguer in Huelva province; and Carchuna, Granada and Torrenueva in Granada province). The random selection allows participants to be representative of the general population of the study area from which they were drawn, thus supporting the generalizability of the results obtained. Children aged 3–11 years registered in these sixteen schools were invited to take part in the study in December 2009. Spanish-speaking children, with at least one parent also Spanish-speaking, living in towns from the selected study area were considered eligible for the study. A total of 496 children met the inclusion criteria and had informed and signed consent by parents or guardians to participate in the study. Authorizations from Ministries of Health and Education of Andalusia were obtained to get access to these schools. The study protocol was approved by the Biomedical Research Ethics Committee of Granada Province.

2.2. Sample collection and DNA extraction

Buccal mucosa cells collection and DNA extraction procedures were carried out according to the method detailed in Freeman et al. (2003) with some modifications. Cotton swabs on wooden sticks were used in schools to remove cells by scraping the inside of the mouth. These swabs were then placed in Slagboom buffer (100 mM NaCl, 10 mM Tris–HCl pH 8.0, 10 mM EDTA pH 8, 0.5% wt/vol SDS) with Proteinase K (0.2 mg/mL). After three weeks storage at room temperature, DNA was extracted. Proteins were removed using ammonium acetate (2M final concentration), and DNA was precipitated with isopropyl alcohol. The DNA was re-suspended in 500 μl of Tris-EDTA (TE) buffer and stored at −20 °C.

DNA purity and concentration were assessed by UV absorbance at 260 and 280 nm using Infinite® 200 NanoQuant (Tecan, Switzerland), and samples were considered to be sufficiently pure if the ratio of absorbance at 260 and 280 nm was 1.8 ± 0.31 (Santella, 2006). The mean DNA concentration found was 67 ± 91 μg/mL. A total of 10 or 20 ng of dried-down genomic DNA was used per reaction for SNP and CNV assays, respectively.

2.3. Genotyping

Hydrolysis probes were used for the detection of eight specific SNPs and two copy number variants (CNVs) by using TaqMan® Assay-based real-time PCR. Specific TaqMan® Predesigned SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) were used: C___2411904_20 (BCHE D70G, dbSNP: rs1799807), C__27479669_20 (BCHE A539T, rs1803274) and C__11708905_10 (PON1 −108C/T, rs705379). TaqMan® Drug Metabolism Genotyping Assays (Applied Biosystems, Foster City, CA, USA) were also used: C___2259750_20 (PON1 L55M, rs854560), C___2548962_20 (PON1 Q192R, rs662), C__25986767_70 (CYP2C19 G681A, rs4244285), C__27102431_D0 (CYP2D6 G1846A, rs3892097). The protocols specifically recommended by the manufacturer were followed.

Genotyping of *CYP3AP1 −44G/A* (rs2177180) polymorphism was performed using Custom Taqman® SNP Genotyping assays (Assay-by-Design) (Applied Biosystems, Warrington, Cheshire, UK), which consist of a mix of unlabeled polymerase chain reaction

(PCR) primers and the TaqMan® minor groove binding group (MGB) probe (FAM™ and VIC® dye-labeled). The following primer sequences were used: forward primer 5′-GTGTGTGCGATTCTTTGC TACTG-3′ and reverse primer 5′-GGTCAAGCTGCTGAAATGTT TATGT-3′, together with a specific TaqMan® FAM™ (5′-AGCCCC**G**CCTCCT-3′) and VIC® (5′-CAGCCCC**A**CCTCCT-3′) dye-labeled MGB probes.

GSTM1 and GSTT1 genetic polymorphisms were determined using pre-designed Taqman® Gene Copy Number Assays designed by Applied Biosystems (Foster City, CA), Hs03947236_cn and Hs00010004_cn (Life Technologies, USA), respectively. The assay was performed as a quantitative duplex PCR assay using gene-specific primers and a gene-specific FAM™-MGB probe along with primers for the RNase P gene and VIC® dye-labeled TAMRA™ probe as the reference gene (TaqMan® Copy Number Reference Assay RNase P, Life Technologies, USA). RNase P is recommended as the standard reference gene in CNV experiments (Mayo et al., 2010). To determine the copy number for the targets in each sample, the real-time PCR data were exported into the CopyCaller® Software (Life Technologies) that provides the calculated copy numbers and predicted copy numbers for each test sample (Grimholt et al., 2014).

As quality control, each series of amplifications included three negative controls without DNA template (no template controls, NTCs) to test for DNA contamination. Each run also included three positive controls with samples of the known genotypes at the CNVs and SNPs of interest, the latter being previously confirmed by DNA sequencing. In the case of CNVs, all samples were amplified by triplicate and for Taqman® SNPs Genotyping Assays, 10% ($n = 50$) of randomly selected samples were analyzed.

Samples were run on the Applied Biosystems 7900HT Fast Real-Time PCR system. Genotyping was performed using Sequence Detection Software (SDS, version 2.4).

2.4. Statistical analysis

The program package ARLEQUIN v. 3.0 (Excoffier et al., 2005) was used for estimating allelic, genotypic and haplotypic frequencies and also to test for the Hardy–Weinberg equilibrium (HWE) expectations. The Bonferroni correction for independent multiple comparisons was used to avoid a type I error. Linkage disequilibrium (LD), which shows the linkage between pairwise SNP combinations on the same chromosome, was studied for PON1 and BCHE polymorphisms. LD parameters (P, D' and r^2) were estimated by using the software package Haploview 4.2 (Barrett et al., 2005). Chi-square test was applied to ascertain significant associations between (a) different genetics polymorphisms in the same gene; (b) differences in frequencies of specific genotypes between males and females and (c) differences in allele frequencies between our study population and those reported in other European and Caucasian populations. This test was performed with the SPSS statistical software package (SPSS 20.0 for Windows). A p-value of 0.05 was adopted as a significance limit.

3. Results

Genotype and allele frequencies of the eight SNPs and two CNVs studied in the selected genes coding for the OP pesticides-metabolizing enzymes are listed in Table 1. The selected genetic polymorphisms studied were in Hardy–Weinberg equilibrium (HWE), although Bonferroni correction was needed for the *PON1 L55M* polymorphism.

Linkage disequilibrium (LD) analysis data for SNPs studied in *PON1* and *BCHE* genes are depicted in Table 2 and Fig. 1. The coding region polymorphism *PON1 Q192R* was in linkage disequilibrium with *PON1 L55M* and the promoter region polymorphism *−108C/T*. *PON1 L55M* also was in linkage disequilibrium with *PON1 −108C/T*. Highest pairwise D' estimates for LD were 0.844 and 0.547, indicating dependence of genetic polymorphisms affecting PON1. Despite these relatively high D' values suggest strong LD, no tag SNPs can be derived from *PON1* locus because of their low r^2 values (0.222 and 0.178, respectively) and r^2 is more sensitive as it depends on the allele frequencies of two loci under consideration. Thus, none of those *PON1* polymorphisms can replace each other.

Chi square analysis of genetic polymorphisms showed statistically significant associations between the two SNPs studied in the *BCHE* gene and between the three *PON1* SNPs studied (data not shown), very likely as a result of their linkage disequilibrium. For the CYP450s studied, significant associations were observed between *CYP2C19* and *CYP3AP1* polymorphic variants. In turn, no association was found between *GSTM1* and *GSTT1* CNVs (data not shown).

Frequencies for combined genotypes with an increased theoretical risk from OP pesticides exposure because of unfavorable versions resulting in higher concentrations of active metabolites are shown in Table 3. For *BCHE*, almost one third of the study population (34.7%) exhibited SNPs combinations other than UU/UU, which corresponds to the wild-type (or usual) genotypes for *BCHE-K*/*BCHE-A* polymorphisms. As regards *PON1* loci, 11.7% presented the apparent worst SNPs combination for metabolizing OP pesticides (*PON1 MM/TT*). In relation to CNVs for GSTs, the combination of null genotypes for *GSTM1* and *GSTT1* was found in 7.1% of the study population. When all genetic polymorphisms studied were considered together, the worst genotype combination (unusual *BCHE*, *PON1 MM*/*TT* and the combined *GSTM1* and *GSTT1* null genotype) was observed in one individual (0.2% of the study population). When CYP450s were assessed for worst genotype combinations, only 2 individuals carried risky genotypes: *CYP3AP1 −44GG* and *CYP2C19 681AA*, which correspond to extensive and poor metabolizers, respectively. Although both individuals also carried the *GSTM1* null genotype, they failed to present a risky genotype for *PON1*, *BCHE* or *GSTT1*.

Table 4 shows haplotype frequencies for the *BCHE* and *PON1* gene polymorphisms studied. Haplotypes for *BCHE* genetic variants A and K as well as for *PON1 Q192R*, *L55M*, and *−108C/T* were determined since multiple linked SNPs have the potential to provide significantly more power for genetic analysis than individual SNPs. This may be useful to determine if any haplotype may increase the associated risk for having an adverse effect from OP pesticide exposure.

We also analyzed our data for possible gender differences in the prevalence of the studied polymorphic gene variants; however, no statistically significant differences were found for any of the genetic polymorphisms studied (data not shown). As for information regarding ethnicity/nationality, 442 children (89.1%) were Spanish, 22 (4.4%) from East Europe (Russia, Romania, Bulgaria), 19 (3.8%) from South America (Argentina, Ecuador, Colombia, Bolivia, Peru), one from Morocco, and 12 children had unknown or missed information. Thus, 54 children (10.9%) of the study population were from countries other than Spain and up to 32 were of non-Caucasian ethnic origin. When Spanish children were compared with non-Spanish children for all the SNPs and CNVs studied, no statistical significant differences were observed except for GSTM1 ($p = 0.025$), as Spanish children had a higher proportion of homozygous for the null genotype (*GSTM1*0*) than non-Spanish children (54.1% vs. 40.7%, respectively).

4. Discussion

This study provides the first comprehensive data on ten polymorphic variants of genes encoding pesticide-metabolizing

Table 1
Genotype and allele frequency distribution in the study population (n = 496 individuals).

Genetic polymorphism	Genotype	n	Genotype frequency (%)	Alleles	Allele frequency (this study)	HWE p-value	Allele frequency (Europeans) [a]	p-Value [b]
BCHE-K (rs1803274)	UU	328	66.1	U	0.811	0.629	0.813	0.950
	UK	149	30.0	K	0.189		0.187	
	KK	19	3.8					
BCHE-A (rs1799807)	UU	480	96.8	U	0.984	0.999	0.985	0.786
	UA	16	3.2	A	0.016		0.015	
	AA	0	0					
PON1 Q192R (rs662)	QQ	229	46.2	Q	0.687	0.364	0.710	0.315
	QR	223	45.0	R	0.313		0.290	
	RR	44	8.9					
PON1 L55M (rs854560)	LL	188	37.9	L	0.594	0.016 [c]	0.649	0.018
	LM	213	42.9	M	0.406		0.351	
	MM	95	19.2					
PON1 −108C/T (rs705379)	CC	115	23.2	C	0.467	0.254	0.513	0.054
	CT	233	47.0	T	0.533		0.487	
	TT	148	29.8					
CYP2C19 G681A (rs4244285)	GG	368	74.2	G	0.859	0.515	0.852	0.696
	AG	116	23.4	A	0.141		0.148	
	AA	12	2.4					
CYP2D6 G1846A (rs3892097)	GG	336	67.7	G	0.820	0.449	0.809	0.563
	GA	141	28.4	A	0.180		0.191	
	AA	19	3.8					
CYP3AP1 −44G/A (rs2177180)	GG	9	1.8	G	0.117	0.426	0.067	<0.001
	AG	98	19.8	A	0.883		0.933	
	AA	389	78.4					
GSTM1	0–0	261	52.6	0	0.723	0.650	53.1% [d]	0.834
	0–1	195	39.3	1	0.277			
	1–1	40	8.1					
GSTT1	0–0	80	16.1	0	0.425	0.085	19.7% [d]	0.054
	0–1	262	52.8	1	0.575			
	1–1	154	31.0					

[a] The 1000 Genomes Project Consortium (2012).
[b] p-Values for differences in allelic distribution between our study population and Europeans and Caucasian populations.
[c] When using the Bonferroni's correction for ten comparisons, no statistically significant deviation from HWE was observed ($p > 0.005$).
[d] Garte et al. (2001). Average frequency for null GSTs genotypes in Caucasian population is shown (n = 10,514 for GSTM1 and n = 5577 for GSTT1).

Table 2
Linkage disequilibrium (LD) parameters values obtained for pairs of the studied polymorphisms in all children studied.

L1	L2	D'	95% CI	p	LOD	r^2	Dist
PON1 Q192R (*rs662*)	*PON1 L55M* (*rs854560*)	0.84	0.76–0.91	<0.001	32.04	0.222	8638
PON1 Q192R (*rs662*)	*PON1 −108C/T* (*rs705379*)	0.21	0.10–0.31	<0.001	2.72	0.023	16,449
PON1 L55M (*rs854560*)	*PON1 −108C/T* (*rs705379*)	0.55	0.46–0.62	<0.001	24.83	0.178	7811
BCHE-K (*rs1803274*)	*BCHE-A* (*rs1799807*)	0.60	0.26–0.81	0.002	2.05	0.025	57,249

D': normalized LD parameter (values >0.8 indicates strong LD).
95% CI (confidence lower and upper bound on D').
p: significance value for D'.
LOD: log of the odds of there being LD between two loci (values >2 indicates significant LD).
r^2: correlation coefficient between the two loci (values >0.8 indicates strong LD).
Dist: distance in bases between the loci.

enzymes (*BCHE*, *PON1*, *CYP450*, *GSTs*) in a Spanish population living near an intensive agriculture area where a high use of pesticides is made (Gómez-Martín et al., 2015). Few studies have thoroughly examined the common genetic polymorphisms of phase I and phase II biotransformation enzymes involved in the human metabolism of xenobiotics (Bosch et al., 2006). The data obtained in this or similar studies contribute to the current toxicogenetic knowledge on important human pesticide-metabolizing enzymes and may prove to be useful for epidemiological studies to understand better the links between pesticide exposure and potential environmental-related diseases or adverse outcomes (Howard et al., 2010; Sarmanová et al., 2000). Frequencies of mutated alleles found in this study were in accordance with the values reported for other European populations of Caucasian origin (Abecasis et al., 2012; Garte et al., 2001) except for *PON1 L55M* and *PON1 −108C/T*, where our population showed increased allelic frequencies of "risky" alleles (M and T, respectively). Conversely, our population had near-significant decreased frequency of null allele for GSTT1 (Table 1).

The risk of potential health effects related to environmental or occupational exposure to pesticides may be modified by genetic variability in enzymes coding genes involved in the metabolism of these compounds. When individuals are not efficient in the detoxification mechanism, metabolic sub-products accumulate contributing to toxicity. The limited evidence available in animals and humans suggests that individuals with genetic variants of BChE showing reduced activity may be more sensitive to OP toxicity (Lockridge and Masson, 2000; Manoharan et al., 2006). The

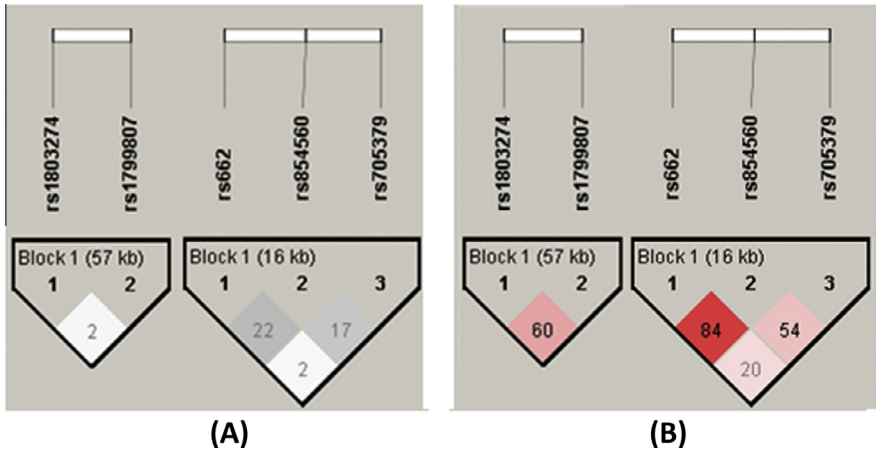

Fig. 1. Pairwise linkage profile of the single nucleotide polymorphisms (SNPs) studied: *rs1803274* (*BCHE-K*), *rs1799807* (*BCHE-A*), *rs662* (*PON1 Q192R*), *rs854560* (*PON1 L55M*), *rs705379* (*PON1 −108C/T*) in all individuals from our study population. The number inside the rhombus indicates the linkage correlation coefficient (r^2) (A) and the LD coefficient (*D'*) (B) obtained from Haploview of the specific linkage of two SNPs. Colors represent r^2 value (shades of gray colors in A) and standard *D'*/LOD (shades of red in B) between the two SNPs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Frequencies of combined genotypes at most risk for adverse effects from OP pesticides exposure.

Combined genotypes	*n*	%
BCHE		
KK/UU	19	3.8
UK/UA	12	2.4
UK/UU	137	27.6
UU/UA	4	0.8
Unusual[a]	172	34.7
PON1		
MM/TT	58	11.7
GST		
*M1*0/T1*0*	35	7.1
CYP450s[b]		
CYP2D6 1846GG	336	67.7
CYP2C19 681AA	12	2.4
CYP3AP1 −44GG	9	1.8

Percentages are given with respect to the total study population ($n = 496$).
[a] Unusual *BCHE* genotypes refer to combined genotypes other than UU/UU for *BCHE-K*/*BCHE-A*.
[b] *CYP2D6 1846GG* and *CYP3AP1 −44GG* are extensive metabolizers while *CYP2C19 681AA* carriers are poor metabolizers.

common *BCHE-K* SNP (K-variant) is associated with ~7% reduction in enzyme activity in heterozygotes and 14% in homozygotes (Podoly et al., 2009). Among the few studies describing the allelic frequencies of *BCHE-K* in different populations based on direct DNA analysis, Benyamin et al. (2011) reported a minor allele frequency of 0.21. Similar frequencies have been observed in populations of different ethnic and geographic origins (Souza et al., 1998) and our results (0.19) are consistent with these studies. Other *BCHE* deficiency variants are uncommon, such as the dibucaine-resistant atypical variant (*BCHE-A*), with an allele frequency from 1.5% to 7.3% in European populations (Acuña et al., 2003; Jensen et al., 1995; Lockridge, 1992). The allele frequency observed in our study (2%) falls within this range. *BCHE-A* variant has been associated with a 33% per-allele decrease in activity compared with the wild-type homozygous state (Bartels et al., 1992). These authors reported that both *BCHE* SNPs are in linkage disequilibrium as also occurred in our study.

The *PON1* gene has two major coding region polymorphisms: *PON1 192Q/R* (which affects the catalytic activity of PON1 in a substrate-specific manner) and *PON1 55L/M* (associated with the serum concentration of the enzyme). However, the main contributor to variation of PON1 levels in serum is the regulatory region polymorphism *PON1 −108C/T*, as it affects PON1 expression (Richter et al., 2009). As all these polymorphisms affect the rate of hydrolysis of toxic oxons of OPs, genotypic characterization of *PON1* gene represents a potential predictor for susceptibility to OP-related effects (Costa et al., 2013).

Other important polymorphisms for pesticide metabolism, particularly for the better known OP biotransformation pathways, are those of the genes coding for CYP2C19, CYP2D6, CYP3A4/5 and CYP2B1, which can result in toxic metabolites or detoxified products (Bozina et al., 2009). These CYP450s show important genetic polymorphisms affecting xenobiotic-metabolizing activity as well as a considerable variability in the allele distribution among different ethnic groups, resulting in variable percentages of poor, intermediate and extensive metabolizers in a given population (Zhou et al., 2009; Zhu et al., 2002). Diminished rates of clearance as a result of low CYP450 activity can increase the incidence of toxicity from many environmental toxicants. For CYP2D6, the most frequent inactivating polymorphism in Caucasians is the *CYP2D6 1846AA* genotype. As 5–14% of Caucasians lack CYP2D6 activity, they are considered poor metabolizers (Bozina et al., 2009). For

Table 4
Haplotype frequencies for *BCHE* gene polymorphisms (rs1803274 and rs1799807) and *PON1* gene polymorphisms (rs662, rs854560 and rs705379).

BCHE[a]			
K variant rs1803274 (A539T)	Atypical variant rs1799807 (D70G)		Frequency
G	A		0.806
A	A		0.178
A	G		0.011
PON1[b]			
Q192R rs662	*L55M* rs854560	*−108C/T* rs705379	Frequency
A	T	T	0.317
A	A	C	0.221
G	A	C	0.160
G	A	T	0.134
A	A	T	0.078
A	T	C	0.070
G	T	C	0.015

[a] Nucleotides C and T in *BCHE-K* variant corresponds to usual (wild-type) and K alleles, respectively. Nucleotides T and C in *BCHE-A* variant correspond to usual (wild-type) and A (atypical) alleles, respectively.
[b] Nucleotide T in the *PON1* gene polymorphisms corresponds to allele Q (for *PON1 Q192R*), allele M (for *PON1 L55M*) and T (for *PON1 −108C/T*).

CYP2C19, the poor metabolizer phenotype occurs in 2–5% of Caucasians and derives from the *CYP2C19 681AA* genotype (Goldstein et al., 1997).

CYP3A5 is the primary extrahepatic CYP3A isoform and its polymorphic expression may be implicated in disease risk because of the metabolism of xenobiotics in tissues other than the liver (Lamba et al., 2002). While a very frequent SNP of *CYP3A5* (*CYP3A5 A6986G*) is widely detectable in Caucasian populations with a G allele frequency of 90% (Lamba et al., 2002), homozygosity for this allelic variant is strongly correlated with decreased CYP3A5 protein expression and enzyme activity in the intestine and liver (Wang et al., 2010). The pseudogene *CYP3AP1*, present in the human *CYP3A* gene cluster, shows a SNP at position −44 (*rs2177180*) which is strongly associated with expression of CYP3A5 enzyme (Kuehl et al., 2001). Individuals homozygous for −44A allele have lower CYP3A activity than heterozygous −44GA, whereas homozygous for −44G allele show the highest activity (Zhu et al., 2002). The close association between *CYP3AP1 −44A* and *CYP3A5 6986G* alleles results from a strong linkage disequilibrium and complete concordance between them, allowing *CYP3AP1* genotypes to be used as an indicator of CYP3A metabolic activity (Zhu et al., 2002). The frequency of the *CYP3AP1 −44A* allele in European populations is 0.933 (1000 Genomes Project Consortium, 2012), very close to that found in our study (0.883). As the mutated *CYP3A5 6986G* and *CYP3AP1 −44A* alleles are not functional against xenobiotics (including pesticides), they result in decreased levels of active metabolites because of reduced bioactivation rates. Thus, carriers of the non-functional alleles are less vulnerable to the toxic effects of these active metabolites (e.g. the oxon metabolite of OPs).

GSTs, in addition to detoxifying reactive intermediates, also play a role by dealkylating methyl or ethyl groups (e.g., methyl parathion and parathion, respectively) and dearylating OPs (e.g., diazinon) (Fujioka and Casida, 2007). As null genotype for these genes may confer higher susceptibility to disease and disease outcome from chemical exposures (Rossini et al., 2002), the determination of *GSTM1* and *GSTT1* genotype polymorphism may be helpful as an individual marker of susceptibility in OP-exposed population (Singh et al., 2011).

Inter-individual variation in adverse effects due to an individual's genetic background cannot be explained only by genetic polymorphisms. Linkage disequilibrium and haplotype analysis may be as well of help to identify the functional significance of polymorphisms. In our study, frequencies for combined genotypes with greater risk from exposure to pesticides are shown in Table 3. In the case of OP exposure (the pesticide group better known for metabolic pathways) about 35% of our study population has genotypes other than UU/UU for the SNPs combination *BCHE-K*/*BCHE-A*. Since carriers of genetic variants of these SNPs have lower BChE catalytic activity, a theoretical increased risk for developing adverse outcomes can be predicted from exposure to anticholinesterase insecticides. Although both SNPs are in linkage disequilibrium (in fact no individual with the KK genotype carried the A allele), no tag polymorphism was found, indicating that both SNPs are irreplaceable and thus they show cumulative effects on enzyme activity. Thus, a 35% prevalence of our target population can be at increased risk from BCHE genetic polymorphisms. Furthermore, 75% of subjects (12 out of 16 children) with genotype UA also had the UK genotype, very likely because of linkage disequilibrium. As regards *PON1* SNPs, 11.7% of our study population presented the genotype combination *MM*/*TT*, which is associated with lower serum protein concentration and activity. While the PON1 *L55M* and *−108C*/*T* polymorphisms showed linkage disequilibrium with each other (Table 2), no tag polymorphism was found, so that the percentage of vulnerable individuals would not be expected to be different from the above frequency. As *PON1 192Q*/*T* polymorphism shows differences in hydrolysis rates and catalytic efficacy (Costa et al., 2013), no assessment can be made on vulnerability to OPs in general, so that any prediction should be made on the basis of individual compounds.

In relation to GSTs polymorphism, 7.1% of the study population have the combination of null genotypes for *GSTM1* and *GSTT1* and thus they are at increased risk for adverse outcomes, particularly from exposure to chemicals (including pesticides) yielding reactive metabolites during their biotransformation. When all genetic polymorphisms studied but CYP450s were considered together, only 1 individual (0.2% of our study population) exhibited the worst genotype combination for pesticide exposure (unusual *BCHE* variants, *PON1 55MM*/*−108TT* and null genotype for both *GSTM1* and *GSTT1*). Predictions for greater susceptibility to pesticide exposure from CYP450s genotype combinations are difficult to conduct as these enzymes are involved in both bioactivation and detoxification of OPs, depending on the type of compound and its concentration. Interestingly, 8 out of the 9 individuals with the genotype *CYP3AP1 −44GG* (which expresses the CYP3A5 protein and thus can activate OPs to oxons) had the genotype *CYP2D6 1846GG* (extensive metabolizers), indicating an increased risk for OP activation, particularly at high environmental doses. From these 8 individuals, 2 had the genotype *CYP2C19 681AA* (poor metabolizers), so they have a limited capacity to detoxify parent OPs before being activated to their corresponding oxons. According to the CYP450 genotype distribution observed in our population, these 2 individuals would have the greatest genetic risk from OP exposure. None of those 8 cases was carrier of a risky *PON1* genotype (*PON1 55MM* or *PON1 −108TT*), one carried the *BCHE-KK* genotype and another one was *BCHE-UK*. From these eight children, none was null for both GSTs and only one was null for *GSTM1*. It may be predicted that by increasing the sample size up to the population level, individuals harboring several unfavorable genotypes for various XMEs would be found.

The possibility of an increased susceptibility due to specific gene mutations is a very complex subject as deficiencies in one particular genotype(s) may be compensated for by other more 'protective' genotypes. The situation is even more complicated for the CYP450 enzymes as they may contribute to both enhanced as well as reduced susceptibility, depending on the actual exposure. As the combination of favorable and unfavorable genotypes of XMEs might have a health impact on long-term exposures, they should be considered as guidance for future studies in order to better improve the prediction of adverse health effects.

The allelic frequency of genetic variants in XME, along with LD and haplotype combination, are important parameters for selecting which SNPs should be analyzed in routine assessment as well as for determining the genetic differences between exposed populations and their risk for developing adverse effects when having the same exposure pattern (Bosch et al., 2006). Furthermore, disease prevention at the individual and population level needs to be evaluated in the context of all the genetic background and exposures to both toxic and beneficial chemicals. Since polymorphisms in the detoxification enzymes that alter protein expression and/or function may modify the risk of toxic response to chemicals (Lampe, 2007), they are increasingly used as toxicological markers of susceptibility to environmental chemicals and to assess gene-environment interactions.

5. Conflict of interest

Authors declare that they have no conflict of interest.

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