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RESEARCH ARTICLE

Associations of xenobiotic-metabolizing enzyme genotypes PON1Q192R, PON1L55M and CYP1A1*2A MspI with pathological symptoms of a rural population in south Greece

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Abstract

1. Paraoxonases and cytochromes P450 constitute two major classes of xenobiotic-metabolizing enzymes involved in the detoxification of pesticide chemicals. In this study, we examined the distribution of two common genetic polymorphisms of the paraoxonase 1 gene and one common polymorphism of the CYP1A1 gene, in relation to pathological diseases occurring in a rural population.
2. Blood and hair samples were collected from 220 participants of an agricultural cohort in the south of Greece for genotype and pesticide analysis. Demographic information and disease status of the participants was obtained by questionnaire, medical examination and medical record. Organochlorine pesticides and metabolites (DDTs, HCHs) were extracted from hair and analyzed using gas chromatography combined with mass spectrometry techniques.
3. Our results indicate exposure of the rural population of Amaliada to organophosphate and past exposure to organochlorine pesticides.
4. Genotypic analysis of PON1Q192R, PON1L55M and CYP1A1*2A MspI polymorphisms was performed using PCR-RFLP. The PON1 192R and 55M alleles absence was significantly associated with hypertension (OR: 2.59; 95% CI: 1.10–6.09) and hepatitis (OR: 21.43; 95% CI: 2.53–181.50), respectively, as indicated from backward logistic regression. Although the presence of PON1 192R allele significantly affected the occurrence of prostate hyperplasia (OR: 0.35; 95% CI: 0.03–0.40), no associations were obtained between the paraoxonase serum activity or the CYP1A1 genotype and the disease status.

Keywords: Paraoxonase 1, cytochrome P450 CYP1A1, polymorphisms, organophosphate pesticides, organochlorine pesticides, associations

Introduction

Pesticides are chemicals used extensively for the protection of the quality of agricultural products. The exposure of rural populations to pesticides has been an active area of research in the field of environmental toxicology in the last years (Dolapsakis et al., 2001; Azmi et al., 2006; Tsatsakis et al., 2008b; Xu et al., 2010). Although acute exposure can be determined in biological matrices such as blood by conventional analytical techniques, assessment of chronic exposure by hair analysis offers unique advantages as opposed to the other biomarker-indicators (Bertsias et al., 2004; Tsatsakis et al., 2008a, 2008b). Chronic exposure to pesticides and insecticides is a matter of increasing concern due to the deleterious long-term toxic effects to the health of the population involved (infertility, neurological symptoms, cancer etc.). Numerous studies have explored the association of organochlorine and organophosphate pesticide levels with the occurrence of health symptoms in the exposed population (Belpomme et al., 2009; Tsatsakis et al., 2009;

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Xu et al., 2010). It is becoming increasingly accepted that the relationship between levels of pesticide exposure and pathological findings is far more complex than initially thought, because of the involvement of several phase I and phase II enzymes that participate in the metabolism and detoxication of xenobiotics (Eaton, 2000; Dehn et al., 2005; Furlong, 2007; Tsatsakis et al., 2009).

The paraoxonases and the cytochrome P450s constitute two major classes of xenobiotic metabolizing enzymes. Paraoxonases are enzymes which are involved in the metabolism of organophosphorous pesticides, whereas cytochrome P450s are implicated in the oxidative metabolism of various drugs and xenobiotics, including organophosphate pesticides (Mutch et al., 2007; Androutsopoulos et al., 2009; Zafiropoulos et al., 2010). The cytochrome P450 CYP1A1 is an extrahepatic enzyme involved in the metabolism of environmental chemicals notably polycyclic aromatic hydrocarbons as well as endogenous and dietary compounds such as oestradiol and flavonoids (Androutsopoulos et al., 2009). Although the duality of this enzyme in the activation or detoxication of carcinogens has been clarified, little is known about its contribution in the metabolism of organochlorine and organophosphate pesticides. It may be assumed that based on the sequence homology with CYP1A2, CYP1A1 could participate in the activation or detoxification of organophosphates, as it has been earlier noted for other CYP isoforms of the first or the second family (CYP2C9, CYP2B6, CYP1A2) (Fabrizi et al., 1999; Albores et al., 2001). A recent report suggests that organophosphates are more effective inhibitors of hepatic CYPs and the extrahepatic CYP1A1, as opposed to other classes of pesticides (Abass et al., 2009). Furthermore, CYP1A1 does not participate directly in the metabolism of some organochlorine pesticides such as DDT and DDE, although it metabolizes polychlorinated biphenyls (PCBs) (Messaros et al., 2009). The most widely examined and characterized biological effect of CYP1A1 is the induction of its expression via the aryl hydrocarbon receptor (AhR), by the prototype CYP1A1 inducers B[α]P and TCDD (Androutsopoulos et al., 2009). This pathway applies for other chemicals including organochlorine pesticides (Dehn et al., 2005; Chan et al., 2009). In addition to environmental, genetic factors are believed to play a role in the expression of the enzyme's catalytic activity. The CYP1A1*2A MspI polymorphism, results from a thymidine to cytosine transition at position 3801 in the 3′ non-coding region downstream from exon 7 of the CYP1A1 gene and has been associated with high inducibility of the CYP1A1 gene (Petersen et al., 1991).

PON1 is one of the three members of the paraoxonase family of enzymes. PON1 resides on circulating HDL and is believed to exert an antioxidant effect by protecting LDL from lipid peroxidation (Mackness et al., 1991; Mertens and Holvoet, 2001). In addition, studies in PON1 knockout mice have demonstrated that PON1 is a key determinant in the detoxification of organophosphate pesticides, such as chloropyrifos (Shih et al., 1998). Two polymorphisms at the coding region of the paraoxonase 1 gene have attracted particular attention due to their effects in the levels of serum PON1 activity. PON1 192 Q/R polymorphism occurs from a Glu/Arg substitution and results in enhanced hydrolysis of paraoxon and chloropyrifos oxon, whereas PON1 55 L/M polymorphism occurs from a Leu/Met amino acid substitution and is thought to be associated with low serum concentration of the enzyme (Zafiropoulos et al., 2010). The protective role of PON1 against pesticide-mediated toxicity is dependent amongst other variables, on the presence of Q and R isoforms in the population. *In vivo* studies have shown that high levels of PON1 activity in serum, due to the presence of the R isoform, are consistent with resistance to toxic pesticide metabolites, such as chloropyrifos oxon (Costa et al., 1990; Costa et al., 2003). Moreover polymorphic variants of PON1 have been examined in terms of their use as biomarkers for susceptibility to certain diseases, notably chronic liver damage, hypertension, and cardiovascular disease (Ferré et al., 2006; Marra et al., 2006).

Work in our research laboratory has demonstrated the use of hair as a powerful indicator of chronic exposure to pesticides that offers a unique link between pathology and exposure assessment compared with other indicators such as blood and urine (Tsatsakis et al., 2008a, 2008b). In addition, a recent cross-sectional study undertaken by our group examined the relation of PON1 Q/R, PON1 L/M, and CYP1A1 MspI polymorphisms with clinical findings of a Greek rural population professionally exposed to pesticides, as determined by GC-MS hair analysis (Tsatsakis et al., 2009). The area selected was the region Messara in Crete, which composes of a small rural cohort. Significant associations between CYP1A1*2A MspI polymorphism and chronic obstructive pneumonopathy as well as other pathological findings were observed, whereas PON1 Q/R polymorphism was associated mainly with hypertension and PON1 L/M with diabetes (Tsatsakis et al., 2009). In the present study, we examined the genotype frequencies of PON1 Q/R, PON1 L/M polymorphisms, in a group of 220 individuals from a rural area of Amaliada, a region located in the Peloponnesus. Amaliada is considered a similar rural region to Messara, in terms of size, where organophosphate pesticides are extensively used. Our results indicate an association of the polymorphic variants with several pathological disorders.

Materials and methods

Study design

Participants were selected on the basis of their occupation from the rural region of Amaliada, Peloponnesus, Greece. A total of 220 individuals participated in the study. Hair samples were collected from all the participants, whereas blood samples were collected from 190 participants. The participants were interviewed for medical history. Questionnaires were designed in order to ascertain biographical data, dietary habits, lifestyle behaviours, and medical history. The study was approved

by the Ethics Committee of the University of Crete and written informed consent was obtained from all donors.

Diagnostic criteria

Laboratory investigations were performed to cross-check the information given by the statements of the participants in the questionnaires and medical history interview. Hypertension was confirmed by measurement of blood pressure following a standardized procedure according to Joint National Committee (JNC) 7 guidelines (Chobanian et al., 2003). Fasting LDL measurements of participants were used as criteria for cholesterol levels. Increased cholesterol was defined in the range of 3.3–4.9 mmol/L LDL in serum. Very increased cholesterol was defined as a measurement of higher than 4.9 mmol/L. Confirmation of diagnosis of diabetes mellitus was based on a fasting blood glucose concentration of higher than 7 mmol/L or a random glucose concentration higher than 11 mmol/L as described in previous reports (Alberti and Zimmet, 1998). Hepatitis A was diagnosed using an ELISA assay for anti-HAV antibodies (Innovative Research, Novi, MI, USA). Therefore, the term hepatitis A positive in our study refers exclusively to the presence of IgG antibodies and not current infection. PSA measurements were also conducted and positive results included participants who showed a free PSA level of higher than 4.0 ng/ml and a ratio of Free PSA/Total PSA of lower than 2.0 (Elabbady and Khedr, 2006). These cutoff values indicate a population subgroup that shows a potential risk of developing prostate-associated hyperplasia or cancer. It should be taken into consideration that our approach was not adequate to point out cases of hypertension, diabetes, and dyslipidemia which were controlled by therapy.

Hair sample collection and pesticide analysis by GC-MS

Hair was removed from the back of the head of the population participating in the study and cut in small pieces (1 mm). Organochlorine pesticides (lindane, α-HCH, HCB, opDDE, ppDDE, opDDT, ppDDT, opDDT and ppDDT) were extracted after incubation of cut hair in 3 N HCl for 12 h at 40°C. The samples were extracted by liquid–liquid extraction using hexane-dichloromethane and final cleanup on solid phase extraction columns. The final eluate was evaporated to dryness and measured by GC-MS (Tsatsakis et al., 2008a). The non-specific metabolites of organophosphate pesticides (DAPs) were extracted from pulverized hair by 2 ml of methanol in an ultrasonic bath for 4 h and liquid–solid extraction was performed by mechanical shaking. Then the mixture was centrifuged, the supernatant was transferred through a filter to a test tube containing 15 mg of K_2CO_3 and methanol was evaporated to dryness. Fifteen milligrams of K_2CO_3, 1 ml of acetonitrile and 0.1 ml solution of pentafluorobenzylbromide (PFBBr) in acetonitrile (1:3 v/v) were added, and the mixture was incubated at 80°C for 30 min. The acetonitrile was evaporated to dryness and 50 μl of toluene were added (Tsatsakis et al., 2010). Analysis was performed by gas chromatography coupled to electron ionization mass spectrometric analysis on a GC MS QP-2010 Shimadzu system equipped with a HP-5MSI or a BPX5 capillary column. Pure helium was used as a carrier gas. Two microliters of the solution were injected into the system in the splitless mode. For DDTs and HCHs: The column temperature was initially held at 60°C for 1 min, raised to 180°C at 15°C/min, held for 1 min, raised to 250°C at 4°C/min, held for 1 min and was finally raised to 300°C, at 30°C/min, where it remained stable for 2 min. For DAPs, the column temperature was initially held at 60°C for 1 min, raised to 180°C at 20°C/min, held for 1 min, raised to 250°C at 4°C/min, held for 1 min and was finally raised to 300°C, at 25°C/min, where it remained stable for 2 min. The injector and interface temperatures were 270°C and 310°C, respectively. The ion source temperature was set at 200°C for organochlorines and at 230°C for DAPs analysis. The mass spectrometer was operated at the selected ion-monitoring mode.

DNA extraction from blood and genotyping

Samples from peripheral blood were stored at 4°C with anticoagulant prior to DNA extraction. DNA was extracted according to previously published methods. PCR was carried out in the presence of two negative controls. The primer sequences used for PON1 L/M, PON1 Q/R and CYP1A1 T/C wild-type and polymorphic variants were based on our previous study (Tsatsakis et al., 2009). The reaction was carried out in the presence of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 mM and 1.2 units of Taq DNA polymerase and the steps included denaturation at 94°C for 3 min, 35 cycles of 92°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final extension step of 10 min at 72°C. The PCR product was then digested with the appropriate endonucleases at 37°C for 4 h, as described previously (Tsatsakis et al., 2009). Digested fragments were visualized by agarose gel electrophoresis (2%) and ethidium bromide staining.

Paraoxonase activity

Serum PON1 activity using paraoxon as a substrate was measured as described in previous methodologies (López-Flores et al., 2009). Paraoxonase activity was determined spectrophotometrically at 412 nm by the release of p-nitrophenol at 25°C, using the molar extinction coefficient 18 mM-1cm-1. Incubations included paraoxon (1 mM), $CaCl_2$ (1 mM) in glycine buffer (pH 10.0). Units of activity were in nmol of p-nitrophenol/min/ml.

Statistical analysis

Discrete variables such as allele frequencies are expressed as counts and proportions. Odds ratios (ORs) were presented in the form of OR (95% CI [confidence interval]). Crude ORs were used to estimate associations between diseases and factors such as age group, sex, occupational variables and polymorphisms expression. Multiple logistic regression models with backward

selection were applied in order to associate diseases with measured variables (age, sex, occupation, polymorphisms expression, presence of pesticides). Simple linear regression was used to estimate correlation between continuous or ordinal variables. Association of PON1 activity with diseases was adjusted with age, sex, smoking and occupation by applying multiple linear regression models. Corresponding association of pesticide levels with diseases was adjusted with age, sex, and occupation. PASW 18.0 was used for statistical analysis.

Results

Characteristics of the population

Table 1 summarizes the demographic characteristics, disease status, and polymorphisms expression of the participants. The study population had a representative portion for the area examined of men and women agricultural workers. The mean age (standard deviation) of participants was 54.6 ± 14.3 years old. The population was divided into three major groups. One group ($n = 122$) was composed of farmers (denoted as active farmers). The second group ($n = 48$) consisted of participants that were not farmers but participated in agricultural works systematically (rural farmers performing agricultural works). The third group was composed of 50 residents that were sparingly or not directly involved in agricultural works (rural residents). Hypertension was a prevalent symptom in both men and women, followed by prostate hyperplasia, diabetes and ulcer that exhibited a lower percentage within the study population (9.5%, 8.0% and 8.0%, respectively). A small percentage of the population was diagnosed positive to hepatitis A. Approximately half of the participants had increased cholesterol levels, whereas other symptoms such as Parkinson's occurred in the population at frequencies below 2% (data not shown).

Documentation of pesticide levels in the study group

The levels of organochlorine pesticides in the hair samples of the entire population of participants in Amaliada have been reported in our previous study (Tsatsakis et al., 2008b). Limits of detection and quantification ranged from 0.5 to 2.5 ng/ml and from 2.5 to 5 pg/mg of hair, respectively. In addition to our previous study, in the present analysis, we determined the prevalence of organochlorine (DDTs and HCHs) and non-specific metabolites of organophosphates (DMP; dimethylphosphate, DEP; diethylphosphate, DETP; diethylthiophosphate and DEDTP; diethyldithiophosphate) in the following three groups of the population: (a) active farmers ($n = 122$); (b) rural residents performing agricultural works ($n = 48$); and (c) rural residents ($n = 50$) (Table 1). OP exposure refers to the above mentioned non-specific organophosphate metabolites. The organochlorine pesticides are the a-HCH, lindane, HCB (HCHs) and the opDDE, ppDDE, opDDD, ppDDD, opDDT and ppDDT (DDTs). Table 2 represents the total levels of pesticide and metabolites found in the hair samples of the entire population in association with occupational exposure or agricultural exposure. The results indicate that there were no significant differences between occupational or agricultural-activity exposure. When the levels of pesticides were compared with disease occurrence significant associations were only observed in the case of hepatitis vs. total DDT ($p = 0.042$), and prostate hyperplasia vs. DMP ($p = 0.003$) whereas a tendency was revealed between hypertension and total DDT levels ($p = 0.082$) and diabetes vs. DMP ($p = 0.052$) (Table 3).

PON1 allele frequencies and correlations with disease symptoms

A total of 190 individuals from the region of Amaliada were genotyped for the presence of PON1 Q192R, PON1L55M and CYP1A1*2A polymorphisms. Table 1 indicates the genotype frequencies of the PON1 and

Table 1. Demographics, disease prevalence and percentages of genotype frequencies of PON1 and CYP1A1 polymorphisms in the entire population.

Demographics	*N* (%)	Diseases	*N* (%)
Sex			
Female	62 (28.2)	Hypertension	57 (29.1)
Male	158 (71.8)	Cholesterol	78 (47.0)
Age (years old)		Diabetes	16 (8.0)
<40	38 (17.4)	Hepatitis	12 (6.0)
40–54	67 (30.6)	Ulcer	16 (8.0)
55–69	80 (36.5)	Angina pectoris	6 (3.0)
70+	34 (15.5)	Prostate hyperplasia (men)	15 (9.5)
Occupation			
Farmers	122 (55.5)		
Agricultural works	48 (21.8)		
Rural residents	50 (22.7)		
Polymorphisms	Wild type (%)	Heterozygotes (%)	Homozygotes (%)
PON1L55M	LL (38.2)	LM (47.3)	MM 14.5
PON1Q192R	QQ (56.0)	QR (37.5)	RR (6.5)
CYP1A1*2A (MspI)	TT (75.9)	TC (23.3)	CC (0.8)

Table 2. Levels of pesticides (median and 1st, 3rd quartiles) vs. occupational group.

	Occupation	*N*	1st quartile	Median	3rd quartile	*P*	*p* (log)
DMP	Rural residents	15	132.7	273.0	1240.0	0.625	0.804
	Agricultural works	42	169.9	409.7	1084.8		
	Farmers	12	148.1	471.3	819.6		
SUMDEP	Rural residents	16	217.9	470.8	863.5	0.509	0.317
	Agricultural works	46	313.7	596.7	1362.1		
	Farmers	13	233.8	376.7	964.6		
SUMDDT	Rural residents	50	<LOQ	<LOQ	6.1	0.702	0.708
	Agricultural works	122	<LOQ	<LOQ	6.2		
	Farmers	48	<LOQ	<LOQ	2.0		
SUMHCH	Rural residents	50	<LOQ	<LOQ	103.5	0.191	0.454
	Agricultural works	122	<LOQ	5.5	117.6		
	Farmers	48	<LOQ	6.3	225.0		

LOQ: Limit of Quantification. SUMDEP: DEP, DETP, DEDTP. SUMDDT: opDDD, ppDDD, opDDE, ppDDE, opDDT, ppDDT. SUMHCH: a-HCH, lindane, HCB. *p*-values as resulted from one-way ANOVA. *p* (log) *p*-values as resulted from one-way ANOVA (log concentration).

Table 3. Levels of pesticides (median and 1st, 3rd quartiles) vs. diabetes, prostate hyperplasia, hypertasis, hepatitis A.

	No				Yes					
	N	1st quartile	Median	3rd quartile	*N*	1st quartile	Median	3rd quartile	*p* (*p*-log)	Adjusted *p* (*p*-log)
Diabetes										
DMP	7	152.3	380.5	861.3	56	344.7	499.9	3330.7	0.052 (0.110)	0.102 (0.276)
SUMDEP	7	311.8	482.4	977.0	62	279.8	627.9	2986.7	0.116 (0.488)	0.219 (0.674)
SUMDDT	16	<LOQ†	<LOQ	5.5	184	<LOQ	<LOQ	6.0	0.486 (0.544)	0.339 (0.313)
SUMHCH	16	<LOQ	<LOQ	121.0	184	<LOQ	11.6	88.1	0.930 (0.323)	0.884 (0.386)
Prostate hyperplasia										
DMP	9	148.8	443.9	752.2	39	303.8	1240.0	2527.5	0.003 (0.070)	0.005 (0.605)
SUMDEP	11	341.6	498.9	1144.7	40	183.7	490.9	1650.9	0.867 (0.803)	0.517 (0.582)
SUMDDT	143	<LOQ	<LOQ	5.6	15	<LOQ	<LOQ	6.8	0.517 (0.639)	0.157 (0.675)
SUMHCH	143	<LOQ	<LOQ	112.9	15	<LOQ	10.2	127.7	0.595 (0.727)	0.522 (0.928)
Hypertasis										
DMP	20	144.9	394.5	846.7	42	272.4	381.3	1178.2	0.371 (0.255)	0.723 (0.109)
SUMDEP	22	330.9	493.6	1119.1	46	312.1	544.9	1010.3	0.795 (0.983)	0.571 (0.768)
SUMDDT	139	<LOQ	<LOQ	6.3	57	<LOQ	<LOQ	5.1	0.082 (0.042)	0.286 (0.260)
SUMHCH	139	<LOQ	<LOQ	117.5	57	<LOQ	<LOQ	122.9	0.845 (0.765)	0.802 (0.922)
Hepatitis A										
DMP	59	165.5	385.7	839.4	4	65.1	760.4	1608.2	0.906 (0.298)	0.898 (0.881)
SUMDEP	65	321.6	490.9	939.3	4	190.8	880.4	1788.8	0.885 (0.874)	0.886 (0.881)
SUMDDT	188	<LOQ	<LOQ	5.5	12	<LOQ	<LOQ	39.9	0.042 (0.053)	0.028 (0.092)
SUMHCH	188	<LOQ	3.4	126.2	12	<LOQ	<LOQ	14.5	0.203 (0.874)	0.208 (0.891)

p (*p*-log): Resulted from simple linear regression on measured concentrations (*p*-value) and log-concentrations (*p*-log). Adjusted *p* (*p*-log): Resulted from multiple linear regression after adjustment of age, sex, occupation. Prostate hyperplasia is adjusted only for age and occupation.
†LOQ: Limit of quantification.

CYP1A1 polymorphic variants. Tables 4 and 5 represent the prevalence of diseases (hypertension, diabetes, hepatitis, prostate hyperplasia) and various parameters amongst categories (sex, age, agricultural works, occupation, DDTs, HCHs levels and PON1 and CYP1A1 polymorphisms). Reported ORs (crude) in Tables 4 and 5 were used to establish associations between diseases. Genotype allele statistical analysis has been performed on the basis of the presence compared with the absence of polymorphic and non-polymorphic alleles and the presence of heterozygotes compared with the presence of homozygotes. Standard polymorphic nomenclature (LL, LM, MM and QQ, QR, and RR for PON1 and TT, TC, CC for CYP1A1) is also indicated in Tables 4 and 5 for clarity. Table 6 indicates the associations of PON1 genotypes with disease occurrence according to *p* values and ORs. Twelve participants from the total population were infected with hepatitis A. In this subgroup, genotype of PON1 polymorphisms revealed that one individual was homozygous and one heterozygous for PON1L55M genotype, whereas the remaining 10 individuals were wild type (Table 4). In contrast, a greater proportion of the participants positive for hepatitis A was found heterozygous for Q192R polymorphism (7 out of 12 individuals), whereas no homozygotes for this genotype were observed (Table 4). Statistical analysis showed an increased OR

Table 4. Prevalence and estimated crude OR's (odds ratio's) for hepatitis A and prostate hyperplasia.

	Positive on hepatitis A		Positive on prostate hyperplasia	
	N (%)	Crude OR (95% CI)	*N* (%)	Crude OR (95% CI)
Sex				
Female	3 (5.7)	1.00	—	—
Male	9 (6.1)	1.09 (0.28–4.18)	—	—
Age				
Years (Mean ± SD)	58.9 ± 10.1	1.03 (0.98–1.07)	68.9 ± 6.9	1.13 (1.06–1.20)
Agricultural works—Occupation				
Rural residence	2 (4.4)	1.00	4 (12.9)	1.00
Agricultural works	9 (7.8)	1.83 (0.38–8.80)	10 (11.2)	0.85 (0.25–2.95)
Farmers	1 (2.5)	0.55 (0.05–6.32)	1 (2.6)	0.18 (0.02–1.73)
SUMDDT				
Negative	8 (6.1)	1.00	9 (8.5)	1.00
Positive	4 (5.9)	0.97 (0.28–3.34)	6 (11.5)	1.41 (0.47–4.19)
SUMHCH				
Negative	9 (8.8)	1.00	7 (8.8)	1.00
Positive	3 (3.1)	0.33 (0.09–1.24)	8 (10.3)	1.19 (0.41–3.46)
DMP (log)				
Mean (SD)	2.92 (0.44)	3.92 (0.29–52.80)	2.92 (0.51)	6.07 (0.81–45.50)
SUM DEP (log)				
Mean (SD)	2.77 (0.53)	1.22 (0.11–13.59)	2.73 (0.45)	0.81 (0.16–4.18)
PON1 L55 polymorphisms				
(LL + LM) vs	11 (7.8)	1.00	13 (9.2)	1.00
(MM)	1 (4.2)	0.52 (0.06–4.21)	1 (4.2)	0.50 (0.06–4.05)
(MM + LM) vs	2 (1.9)	1.00	9 (8.8)	1.00
(LL)	10 (15.8)	9.74 (2.06–45.94)	5 (7.9)	0.95 (0.30–3.01)
(MM + LL) vs	11 (12.6)	1.00	6 (6.9)	1.00
(LM)	1 (1.3)	0.09 (0.01–0.69)	8 (10.3)	1.36 (0.44–4.15)
PON1 Q192 polymorphisms				
(QQ + QR) vs	12 (6.9)	RR: 1.00	14 (8.1)	1.00
(RR)	0 (0.0)	0.94 (0.90–0.97)	1 (8.3)	2.27 (0.24–21.73)
(QR + RR) vs	7 (8.6)	RR: 1.00	12 (14.8)	1.00
(QQ)	5 (4.8)	0.47 (0.14–1.54)	3 (2.9)	0.13 (0.04–0.49)
(QQ + RR) vs	5 (4.3)	1.00	4 (3.5)	1.00
(QR)	7 (10.1)	2.61 (0.80–8.54)	11 (15.9)	6.04 (1.81–20.09)
CYP1A1*2A MSP1 polymorphisms				
(TT + TC) vs	9 (6.8)	RR: 1.00	—	—
(CC)	0 (0.0)	0.94 (0.90–0.98)	—	—
(TC + CC) vs	5 (15.6)	1.00	2 (6.3)	1.00
(TT)	4 (3.9)	0.22 (0.05–0.85)	8 (7.9)	1.35 (0.27–6.81)
(TT + CC) vs	4 (3.9)	1.00	8 (7.8)	1.00
(TC)	5 (16.1)	4.87 (1.23–19.32)	2 (6.5)	0.74 (0.15–3.73)

Percentages indicate the proportion of participants in the entire population for each variable (genotype, sumDDT, or occupation) within the hepatitis or prostate hyperplasia subgroup.

(21.43; 95% CI: 2.53–181.50) with participants lacking the L55M polymorphism and positive hepatitis A, although no significant associations were noted for Q192R polymorphism (Table 4). Genotyping for the CYP1A1*2A MspI polymorphism indicated only 5 participants that were heterozygotes for the latter, whereas the remaining 4 participants did not contain the polymorphic variant either in a heterozygous or homozygous state. There were no homozygotes for MspI polymorphism in this subgroup (Table 4).

Genotype analysis of the participants that presented with prostate hyperplasia indicated that 9 out of 14 and 1 out of 14 were heterozygotes and homozygotes for the L55M polymorphism, respectively, whereas 4 participants did not show the presence of the polymorphic variants (Table 4). Similarly, a very small percentage of PONQ192R homozygotes was found (1 in 15 participants), whereas a large number of the total population group were heterozygotes for the Q192R genotype (Table 4). The associations of prostate hyperplasia occurrence, age and PON1 genotypes are shown in Table 4. Increased OR were obtained for PONQ192R variants and prostate (OR: 2.86; 95% CI: 2.50–33.3) including also age in the final model (OR: 1.16; 95% CI: 1.05–1.28).

The hypertension subgroup was examined for the presence of the two PON1 polymorphisms and substantial portion of the participants presented the heterozygote variant for L55M and Q192R polymorphisms (23 in 54 and 27 in 56, respectively). Homozygotes were found at lower frequencies (5 in 54 and 3 in 56 for L55M and Q192R, respectively) (Table 5). In contrast, the CYP1A1*2A MspI polymorphism was found at low frequencies (12 out of 41 participants) in the heterozygotic state and only one homozygote was found (Table 5). Statistical analysis showed increased ORs, between the absence of the Q/R heterozygotic polymorphic variants and the occurrence of hypertension (OR: 2.59; 95% CI: 1.10–6.09) while positive effect was observed with age (OR: 1.10; 95% CI: 1.05–1.14) (Table 5). There were no significant differences noted for MspI polymorphisms and hypertension.

In an effort to identify possible associations between the diabetic participants and CYP1A1 and PON xenobiotic enzyme genotype distribution, the genotype status of the polymorphic variants was compared with disease occurrence. Out of 16 participants that presented with diabetes, only 1 and 2 were homozygous for PON1L55M and PON1Q192R polymorphisms whereas

Table 5. Prevalence and estimated crude OR's (odds ratio's) for diabetes and hypertension.

	Positive on diabetes		Positive on hypertension	
	N (%)	OR (95% CI)	*N* (%)	OR (95% CI)
Sex				
Female	4 (7.5)	1.00	18 (34.6)	1.00
Male	12 (8.2)	1.09 (0.34–3.54)	39 (27.1)	1.43 (0.72–2.81)
Age				
Years (Mean ± SD)	57.9 ± 12.8	1.02 (0.98–1.06)	51.3 ± 13.8	0.94 (0.90–0.96)
Agricultural works—Occupation				
Rural residence	2 (4.4)	1.00	15 (34.9)	1.00
Agricultural works	11 (9.6)	2.27 (0.48–10.69)	31 (27.2)	0.70 (0.33–1.48)
Farmers	3 (7.5)	1.74 (0.28–11.00)	11 (28.2)	0.73 (0.29–1.87)
SUMDDT				
Negative	11 (8.3)	1.00	37 (28.2)	1.00
Positive	5 (7.4)	0.87 (0.29–2.62)	20 (30.8)	0.89 (0.46–1.70)
SUMHCH				
Negative	7 (6.9)	1.00	29 (29.0)	1.00
Positive	9 (9.2)	1.37 (0.49–3.84)	28 (29.2)	0.99 (0.54–1.84)
DMP (log)				
Mean (SD)	2.61 (0.45)	4.23 (0.70–25.74)	2.74 (0.42)	2.02 (0.60–6.78)
SUM DEP (log)				
Mean (SD)	2.85 (0.57)	1.95 (0.30–12.56)	2.75 (0.40)	0.99 (0.29–3.37)
PON1 L55 polymorphisms				
(LL + LM) vs	15 (10.6)	1.00	49 (34.8)	1.00
(MM)	1 (4.2)	0.37 (0.05–2.93)	5 (20.8)	2.02 (0.72–5.67)
(MM + LM) vs	8 (7.8)	1.00	28 (27.5)	1.00
(LL)	8 (12.7)	1.78 (0.64–4.99)	26 (41.3)	0.52 (0.27–1.00)
(MM + LL) vs	9 (10.3)	1.00	31 (35.6)	1.00
(LM)	7 (8.9)	0.82 (0.29–2.30)	23 (29.5)	1.37 (0.72–2.61)
PON1 Q192 polymorphisms				
(QQ + QR) vs	14 (8.1)	1.00	53 (30.8)	1.00
(RR)	2 (16.7)	3.57 (0.68–18.84)	3 (25.0)	0.80 (0.19–3.33)
(QR + RR) vs	8 (9.9)	1.00	30 (37.0)	1.00
(QQ)	8 (7.7)	0.67 (0.24–1.86)	26 (26.0)	1.97 (1.05–3.69)
(QQ + RR) vs	10 (8.7)	1.00	29 (25.2)	1.00
(QR)	6 (8.7)	1.06 (0.37–3.03)	27 (39.1)	0.52 (0.28–0.98)
CYP1A1*2A MSP1 polymorphisms				
(TT + TC) vs	11 (8.3)	RR:	41 (31.0)	RR:
(CC)	0 (0.0)	0.92 (0.88–0.97)	0 (0.0)	0.71 (0.64–0.79)
(TC + CC) vs	4 (12.5)	1.00	12 (37.5)	1.00
(TT)	7 (6.9)	0.50 (0.14–1.82)	29 (28.7)	1.62 (0.71–3.69)
(TT + CC) vs	7 (6.8)	1.00	29 (28.4)	1.00
(TC)	4 (13.0)	2.09 (0.57–7.63)	12 (38.7)	0.58 (0.25–1.34)

Percentages indicate the proportion of participants in the entire population for each variable (genotype, sumDDT, or occupation) within the diabetes or hypertension subgroup.

no homozygotes were found for CYP1A1*2A MspI polymorphisms (Table 5). In contrast, 7 diabetic participants were heterozygous for the L55M and 6 for the Q192R polymorphism (Table 5), whereas only 4 were found for CYP1A1*2A MspI. Statistical analysis indicates a tendency between the presence of Q192R polymorphic variants and the occurrence of diabetes (OR: 4.37; 95% CI: 0.77–24.85).

PON1 paraoxonase activity

In order to examine whether the associations of PON1 genotypes with disease status could be attributed to functional activity of the PON1 enzyme, we measured paraoxonase 1 activity in the participants that were genotyped for PON1 Q/R and PON1 L/M polymorphisms. Paraoxonase activity units were statistically examined in terms of disease occurrence (prostate, hypertension, hepatitis and diabetes), using multiple regression model, in order to elucidate any possible association with pathology (Table 7). The results showed that no statistical differences were observed (Table 7).

Discussion

The aim of the present study was to investigate the disease status of a rural population in a region of south Greece, part of which was exposed occupationally to organochlorine and organophosphate pesticides, in relation to Q/R and L/M, and T/C MspI polymorphic variant occurrence of the xenobiotic metabolizing enzymes PON1 and CYP1A1. The results indicate exposure of the population to organochlorine and organophosphate pesticides and association between PON1 L55M and Q192M polymorphisms and disease status, notably hepatitis A, hypertension and prostate hyperplasia.

The population investigated in the present study was burdened with organochlorine (Tsatsakis et al., 2008b) and organophosphate pesticides. Exposure to organophosphate pesticides such as diazinon and its implication to pathology (e.g., Parkinson's disease) has been shown by recent studies (Firestone et al., 2005; Manthripragada et al., 2010). In addition to reinforcing these data, the present study advances in determining of organophosphate exposure through the study of their non-specific metabolites in hair, thus providing an indication of long-term exposure. So far, organophosphates exposure assessment has been determined in residential areas from usage reports and a geographic information system approach, in blood samples, by questionnaire information, in drinking water, soil and house dust (Akgür et al., 1999; Lu et al., 2004; Firestone et al., 2005; Azmi et al., 2006; Gatto et al., 2009). The data presented in the current study and our previous report (Tsatsakis et al., 2008a) provide the most conclusive evidence, in terms of long-term exposure of total pesticide and metabolite levels (DAP, DDTs, HCHs), whereas the concentrations of pesticides in hair and the percentage of positive samples in the population add insight to the severity of pesticide occupational exposure and use and the underlying pathology and disease symptoms that may occur in the population exposed.

Table 6. Results of multiple logistic regressions for diabetes, hepatitis, hypertension and prostate hyperplasia.

Disease	Variables	OR (95% CI)	*p*
Hepatitis	SUMHCH	0.21 (0.04–1.14)	0.071
	PON1 L55M (MM + LM) vs. (LL)	21.43 (2.53–181.50)	0.005
Diabetes	**PON1 Q192R** (QQ + QR) vs. (RR)	4.37 (0.77–24.85)	0.096
Prostate hyperplasia	Age	1.16 (1.05–1.28)	0.003
	PON1 Q192 (QQ) vs. (QR + RR)	2.86 (2.50–33.3)	0.007
Hypertension	Age	1.10 (1.05–1.13)	<0.001
	PON1 Q192R (QR + RR) vs. (QQ)	2.59 (1.10–6.09)	0.030

ORs and *p*-value estimation were performed using backward selection model with initial variables sex, age, agricultural works, occupation, PON1 and CYP1A1 polymorphisms and exposure.

Table 7. Paraoxonase activity in disease subgroups and association with pathology.

Disease		Mean (SD)	Median	*p*	Adjusted *p*
Prostate hyperplasia	Negative ($n=89$)	249.3 (200.1)	187.4	0.610	0.575
	Positive ($n=10$)	283.1 (174.1)	226.3		
Hypertension	Negative ($n=93$)	245.7 (200.0)	186.0	0.643	0.128
	Positive ($n=35$)	263.3 (164.5)	224.0		
Hepatitis	Negative ($n=123$)	251.7 (193.3)	194.9	0.649	0.412
	Positive ($n=8$)	219.9 (132.0)	212.6		
Diabetes	Negative ($n=124$)	252.7 (194.3)	196.7	0.458	0.789
	Positive ($n=7$)	197.6 (62.4)	220.9		

Results are expressed as mean and median values of units of activity in nmol/min/ml in each disease subgroup. SDs and the number of participants found positive for each disease in the entire population are shown in parentheses. *p*: Resulted from simple linear regression. Adjusted *p*: Resulted from multiple linear regressions after adjustment of age, sex, occupation and smoking. Prostate hyperplasia is adjusted only for age, occupation and smoking.

Currently, there are no international reference values for pesticide levels in hair, as this is an approach that is used from a limited number of laboratories worldwide. Nevertheless, in a recent publication from our group the limit of quantification and limit of detection for the determination of pesticide in a hair-specific assay was estimated at levels of 5–20 pg of DAPs per mg of hair (Tsatsakis et al., 2010). This assay was developed for DMP, DEP, DETP and DEDTP metabolites. Median concentrations were estimated at levels of 51.2 pg/mg for DEPs in the general population group and 812.9 pg/mg of hair for DEPs in the occupationally exposed group. In the current study, organochlorine pesticides were found at considerably low levels (20–80 pg/mg), compared with DAPs (350–600 pg/mg). The assessment of dialkylphosphates burden can be considered as an indicator of direct organophosphate exposure (OP are metabolized to DAPs) as well as indirect exposure due to the levels of organophosphate present in the dietary food and water. In addition, Posecion et al. reported levels of organophosphate pesticides at the ng range (4.85 and 4.58 ng/mg for chloropyrifos and malathion) in hair of pregnant women indicating that higher concentrations of pesticides are possible to be achieved in this biological matrix (Posecion et al., 2006). Organochlorine compounds (HCHs, DDTs) show high lipophilicity and, as a result, are incorporated in the body and released in hair during time via the blood circulation to the hair follicles (Tsatsakis, 2009). Moreover, the organochlorine levels in hair reflect exposure due to the environmental contamination such as dietary food and drinking water. Consequently, organochlorine levels in hair determined in our present study are not associated with exposure via active use of organoclorine pesticides at present but mostly via dietary exposure.

PON1 is a detoxification enzyme that is synthesized in the liver and then released to the circulation where it binds with LDL. As a result, poor liver function attenuates the levels of PON1 in serum. This finding is supported by numerous studies, where a decreased PON1 activity has been observed for patients with chronic liver damage, liver cirrhosis and hepatitis (Ferré et al., 2002, 2005, 2006; Kilic et al., 2005). Of note is that PON1 activity has been proposed as a marker for hepatic function, along with conventional markers such as transaminases and bilirubin (Ferré et al., 2002; Ali et al., 2009). The polymorphism PON1 L55M affects mainly the concentration of PON1 in serum—notably the latter is associated with decreased PON1 mRNA levels—whereas the polymorphism PON1 Q192R produces variants that hydrolyze paraoxon faster than and thus affects predominantly PON1 activity.

To our knowledge, there are very few studies that have examined the association of PON1 L55M and PON1 Q192R polymorphisms with hepatitis. Ferré et al. reported no significant differences between PON1 L55M genotypes in a case control study of 186 participants with chronic hepatitis related to HCV infection and 386 healthy individuals, whereas a significant association was noted for the RR isoform, between the control and the hepatitis group (Ferré et al., 2005). In the present study, participants carrying the L55M variant exhibited a positive association with serum hepatitis A IgG antibodies. This finding has not been reported previously. One possible explanation could be due to the involvement of PON1 to organophosphate metabolism. Organophosphate pesticides are metabolized to their reactive oxons, notably by cytochrome P450 metabolism and detoxified by oxon-hydrolysis by PON enzymes. Exposure to organophosphate pesticides causes several toxic effects such neurological disorders, endocrine control problems and hepatic toxicity. Diazinon has been shown to be associated with increased ALP, AST levels and hepatitis in a group of 287 farm workers (Azmi et al., 2006). Since L55M variants are associated with lower PON1 mRNA and protein levels in serum, this may reduce the ability of the enzyme to detoxify organophosphates. Consequently, carriers of the LM or MM genotype of the organophosphate-exposed population may show a positive association to hepatic damage caused either by viral or chemically induced hepatitis due to decreased organophosphate metabolic turnover in the body.

Polymorphic variants were further examined for their possible association with hypertension in the population studied. The relationship between PON1 activity and PON1 polymorphisms with cardiovascular disease has been the focus of extensive epidemiological investigations. Several studies have supported the notion that the RR genotype is a risk factor for cardiovascular disease (Gupta et al., 2009). Similar findings have been reported for the MM genotype, although results in the literature are contradictory (Ko et al., 1998; Oliveira et al., 2004; Nus et al., 2007; Gupta et al., 2009). It is generally believed that the QQ genotype confers resistance to cardiovascular disease symptoms because it can potentially prolong the oxidation of LDL by free radicals, whereas the MM genotype is associated with a higher tendency to develop cardiovascular-related diseases due to decreased production of serum PON1 enzyme. With regard to hypertension, a recent study highlighted that the PON1192RR genotype contributes an increased risk to the development of arterial hypertension (Marra et al., 2006). Our results may seem to contradict the previously established theory, where it is argued that for the development of arterial hypertension, the RR, rather than the QQ, genotype contributes an increased risk to the disease, as according to our results a protective role of the Q192R polymorphism was found for the occurrence of the disease in 57 participants of the population. This is probably due to the fact that this study did not solely examine the effect of hypertension and PON1 polymorphisms. The population used in the current study was exposed to organophosphate pesticides. As a consequence, hypertension could be the result of pesticide chronic exposure, since some organic pollutants and pesticides have been previously documented to show gender-specific associations with the development of hypertension (Ha et al., 2009). Thus

the presence of PON1Q192R variants may have protective implications in the hypertensive participants, due to rapid elimination or metabolism of organophosphate pesticides, as the R isoform is associated with higher paraoxonase 1 activity.

In addition to hypertension and hepatitis prostate hyperplasia occurrence was associated with PON1Q192R genotype. To date, there is no study in the literature that has examined this type of association. However, there is a paucity of studies in the literature that have examined the associations of PON1 SNPs with prostate cancer. Stevens and colleagues reported in a recent study that only PON1 L/M or Q/R heterozygotes were associated with an increased risk for developing prostate cancer in a case control study with 1,262 cases and 1,266 controls (Stevens et al., 2008). The risk was increased twofold when a combination of Q/R with L/M genotypes was compared with disease status. Antognelli et al. (2005) reported a higher risk of prostate cancer occurrence with participants carrying the RR genotype, whereas carriers of the QQ genotype showed a protective trend against the development of prostate cancer. Our results are in well agreement with the above conclusion. The aetiological factors involved in prostate cancer are unclear although exposure to environmental pollutants, pesticides and other xenobiotics has been suggested as risk factors (Xu et al., 2010). The finding that RR carriers show a decreased risk for developing prostate hyperplasia can be explained by the increased activation capacity of the corresponding PON1 enzymes produced. Q to R substitution to the coding region of the paraoxonase gene leads to an enzyme with higher activity than the wild type and thus higher capacity to activate environmental pollutants and pesticides that can lead to carcinogenic product formation. Generally increased oxidative stress has been proposed by previous studies as the main aetiology that links PON1 genotype status to prostate carcinogenesis.

Our analysis did not reveal a significant association with disease occurrence and paraoxonase activity. PON1 activity in serum has been shown to vary among individuals due to a variety of environmental factors such as dietary habits, alcohol consumption and BMI (Costa et al., 2003, 2005). Hence the lack of association of PON1 with disease might be influenced by such factors, in addition to the genotype frequencies.

No associations were obtained between diabetic participants and xenobiotic metabolizing enzyme variants CYP1A1*2A MspI and PON1L55M. The polymorphism PON1Q192R exhibited a tendency towards the presence of diabetes in a small group of the population. This trend could probably be attributed to the involvement of PON1 with decreased oxidative stress, as diabetes is a disease that co-progresses with increased free radical formation. One recent study has underlined in a population of 51 diabetic patients and 53 controls that the PON155M allele had greater risk for general periodontal and/or gingival problems, which are indicators of the contribution of reactive oxygen species in the progression of the disease (Unür et al., 2008).

No associations with pathology and the presence of CYP1A1*2A MspI polymorphisms were obtained in the present study. Mechanistically evidence is stronger for the contribution of paraoxonase PON1 enzyme to organophosphate metabolism. Despite this fact, we set out to investigate CYP1A1*2A MspI genotype status, as a continuation of our first indication in the study of Messara, that this enzyme may be involved in pathology and pesticide exposure (Tsatsakis et al., 2009). Moreover, Messaros and colleagues found a significant protective effect of CYP1A1*2A MspI polymorphisms in low sperm morphology of participants exposed to DDT-DDE pesticides (Messaros et al., 2009). The interpretation of such results is not direct, since no proven biological basis can be found for the metabolism of DDE or DDTs by CYP1A1, while the lack of metabolism of organophosphates such as diazinon or parathion by CYP1A1 that has been supported by some studies does not provide direct evidence of organophosphate CYP1A1-associated toxicity (Fabrizi et al., 1999; Hreljac and Filipic, 2009). Thus observations that point towards the idea that CYP1A1 is associated with pathology in populations exposed to organochlorine and organophosphate pesticides are supported by indirect effects occurring through the metabolism of CYP1A1 substrates. Consistent with this conclusion is the recent report on B[a]P modulation by parathion and paraoxon, where it is shown that the latter compounds are inducers of CYP1A1, but they decrease its metabolic B[a]P conversion due to competition binding for the receptor AhR (Hreljac and Filipic, 2009).

In summary, in the present study, we have examined the relationship of pathology with genetic polymorphisms of the xenobiotic enzymes PON1 and CYP1A1 in a rural population exposed in the past to organochlorine and organophosphate pesticides as demonstrated by hair analysis. The data demonstrate exposure of the population to organophosphate as well as to organochlorine pesticides yet to much lower quantities (DDTs and HCHs). Furthermore, the data revealed negative association of the PON1Q192R and PON1L55M polymorphisms with hypertension and hepatitis but a positive association with prostate hyperplasia. The exact mechanisms that underlie the pathological conditions and disease symptoms that occur in rural populations, occupationally exposed to pesticides in the past, are still not known probably due to the complexity and multifactorial nature of the parameters that influence the development of such diseases. Despite this fact, the current study provides a focus to specific disease symptoms that are more prone to occur, as a result of chronic exposure to pesticides and genetic background of PON1 genes although further studies are required in order to unravel the underlying causes of pathology to the disease symptoms of prostate hyperplasia and hypertension in a rural population occupationally exposed to pesticides.

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Declaration of interest

The authors declare that they have no competing interests.

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