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GST, CYP and *PON1* polymorphisms in farmers attributing ill health to organophosphate-containing sheep dip

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Abstract

Previously we reported that in sheep dippers exposed to organophosphates the frequency of paraoxonase (*PON1*) polymorphisms differed between those with or without self-reported ill health. We have now examined whether polymorphisms in other genes involved in xenobiotic metabolism alter disease risk in this population. There were elevated but non-significant risks associated with the *CYP2D6 WT* genotype (odds ratio (OR) 1.47, 95% CI 0.83–2.60), or a *GSTP1*B* or **C* allele (OR 1.37, 95% CI 0.88–2.01) or being *GSTM1*2/GSTT1*2* homozygous (OR 1.61, 95% CI 0.74–3.48). Similar results were generally obtained after the exclusion of subjects to obtain a more homogenous case-referent population: for double null *GSTM1* and *GSTT1* homozygotes the OR was 2.06 (95% CI 0.85–2.04). In those also likely to have been exposed to diazinon, risks associated with a *GSTP1*B* or **C* allele (OR 1.82, 95% CI 0.92–3.63) or a *GSTM1*2/GSTT1*2* homozygous (OR 2.60, 95% CI 0.72–10.42) were elevated but not to a significant extent. Risk associated with *PON1* genotype and phenotype varied with *CYP2D6* and *GSTP1* genotype but not consistently with *a priori* hypotheses. Further work is necessary to delineate more clearly pathways of organophosphate activation and non-*PON1* pathways of detoxification and to confirm whether *CYP* and *GST* polymorphisms alter disease risk in populations exposed to organophosphates.

Keywords: *Paraoxonase, organophosphates, sheep dip, PON1, GST, CYP*

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Introduction

Acute organophosphate poisoning can result in significant neuropsychological abnormalities but evidence linking low-dose chronic organophosphate exposure to similar effects is lacking (Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment 1999). In the UK, sheep farmers often complain of chronic ill health which they attribute to repeated exposure to organophosphates

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(OPs) during sheep dipping (Royal College of Physicians of London and the Royal College of Psychiatrists 1998). These symptoms appear to occur only in a small proportion of farmers implying the presence of a susceptible subgroup of individuals (Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment 1999). If organophosphates were associated with chronic ill health, then an important determinant of susceptibility may be interindividual variability in the ability to metabolise and detoxify OPs (Eaton 2000, Furlong et al. 2000).

OPs can be metabolised by a number of different pathways that involve a range of enzyme systems (Dillon & Ho 1987, Jokanovic 2001). Cytochrome p-450 enzymes can mediate a number of different reactions including desulphuration and activation of an inert OP parent compound into an active metabolite (Dillon & Ho 1987, Jokanovic 2001). For example, diazinon, an OP widely used in sheep dip in the UK, is metabolised by a number of different CYPs including CYP3A4 and CYP2D6 into the toxic metabolite diazoxon and other metabolites (Sams et al. 2000). Both enzymes show large interindividual variation in activity (May et al. 1994, Sachse et al. 1997). Variation in CYP2D6 activity is largely genetically determined (Ingelman-Sundberg 2005) and polymorphisms in the *CYP2D6* gene have been associated with a number of different diseases (Wolf & Smith 1999). Variation in CYP3A4 activity may also be genetically determined as polymorphisms in the *CYP3A4* gene have been reported (Lamba et al. 2002) but it is unclear whether these polymorphisms have any functional significance (Westlind et al. 1999).

OP hydrolysis and inactivation may also be mediated by different enzymes but serum paraoxonase (PON1) is a major factor determining OP toxicity to vertebrates including man (Mackness et al. 1998a, Shih et al. 1998, Furlong et al. 2000). In the human there is considerable individual variation in the serum activity of PON1 and this is partly genetically determined (Mackness et al. 1998a, La Du et al. 2001). The human serum *PON1* gene is polymorphic at two sites in the coding region (55 and 192) and the effect of these polymorphisms on the ability of the PON1 protein to metabolise different OPs remains unclear. It is known that this protein metabolises diazoxon and that the *PON1-192 Q/R* polymorphism is associated with altered catalytic efficiency *in vitro* and *in vivo* that is substrate dependent (Davies et al. 1996, Li et al. 2000). Mice with the Q/R isoforms differed in their susceptibility to chlorpyrifos oxon but not diazoxon or paraoxon (Li et al. 2000). The *PON 1-55 L/M* polymorphism is reported to have little effect on catalytic activity (Humbert et al. 1993) but has been associated with variability in plasma levels (Garin et al. 1997, Mackness et al. 1998b).

The importance of other enzyme systems in the removal of toxic metabolites is unclear. Diazoxon may be metabolised further (Dillon & Ho 1987) and it has been reported that glutathione *S*-transferases (GST) may also degrade diazinon or methyl parathion (Motoyama & Dauterman 1980, Jokanovic 2001, Abel et al. 2004) but whether GST-mediated reactions occur *in vivo* has been doubted (Sultatos 1992). *GSTM1, GSTT1* and *GSTP1* are polymorphic genes and interindividual variation in the expression or functional activity of these enzymes may be important in determining susceptibility to human disease (Rebbeck 1997, Strange et al. 2001). Deletions in both the *GSTM1* and *GSTT1* genes result in the total absence of enzyme activity whereas polymorphisms in the *GSTP1* gene have been reported to affect the activity of the enzyme for at least some substrates (Ali-Osman et al. 1997, Rebbeck 1997). The role of GSTP1 in OP metabolism is unclear but patients with Parkinson's

disease who reported exposure to pesticides were more likely to have *GSTP1* non-A alleles than referents (Menegon et al. 1998).

In a previous study, we reported differences in the distribution of *PON1* genotypes between sheep dippers with self-reported chronic ill health as a result of OP exposure, and healthy controls with a similar dipping history (Cherry et al. 2002, Mackness et al. 2003, Povey et al. 2005). As susceptibility to OP poisoning may potentially result from the balance between activation and detoxification systems, and this balance may be different for different exposures, we have further examined the same population for specific polymorphisms in *CYP* and *GST* genes and have also examined whether such differences vary in those farmers with known exposure to a specific organophosphate (diazinon), and in subpopulations after exclusions of subjects on clinical grounds or after discriminant analysis.

Materials and methods

A case-referent study was carried out in which cases were sheep farmers with self-reported chronic ill health which they attributed to sheep dipping. Each case was asked to nominate referents who were not blood relatives, were of the same gender and did not suffer from chronic ill health, but who carried out a similar regime of sheep dipping. Cases and referents were interviewed by a research nurse and asked to provide information on health and an exposure history based upon questionnaires previously used in a Gulf War study (Cherry et al. 2001). A venous blood sample was taken, frozen immediately and taken back to the laboratory where it was stored at −80°C until DNA was extracted. The study had approval from a Local Research Ethics Committee and was carried out in accordance with British regulations.

Health questionnaire

Subjects were asked in detail about their health during the previous month, indicating on an adjacent visual analogue scale ranging from 'not at all' to 'very seriously', how much they had been troubled by each of the 95 symptoms. Subjects who provided usable answers to at least 90 of the 95 symptoms were included in the main analysis. The subject's mean response to all other symptoms was assigned where five or fewer symptoms had been missed (Cherry et al. 2001).

Genotyping and phenotyping

DNA was extracted from whole blood. Genotyping for *GSTM1*1/*2*, G*STT1*1/*2*, *GSTP1*A/*B/*C*, *CYP2D6*3/*4*, *CYP3A4*1A/*1B*, *CYP3A5*1/*3* was carried out using PCR-based procedures (Rebbeck et al. 1998, Sata et al. 2000, Kuehl et al. 2001, Povey et al. 2001, Donn et al. 2002, Lewis et al. 2002). The *CYP2D6* genotype was defined by identifying the *CYP2D6*3* and *2D6*4* alleles. *CYP2D6* wildtype (WT) was defined as having no **3* or **4* allele, *CYP2D6* heterozygotes (HET) as having one **3* or **4* allele and *CYP2D6* homozygotes (HOM) as having two **3* or **4* alleles or one **3* and **4* allele. *CYP2D6 HOMs,* thus defined, have also been classified (Smith et al. 1992) as *CYP2D6*-poor metabolisers (PM). Genotyping for the *PON1-55* and the *PON1-192* polymorphisms was carried out as described previously (Cherry et al. 2002, Mackness et al. 2003). Serum PON1 activity towards diazoxon was analysed spectrophotometrically as described previously (Davies et al. 1996).

Exposure assessment

Every pesticide reported to have been used, by each farmer, for the period 1970–2000 was extracted on to a database. All 1500 reported 'pesticides' were decoded, as far as was reasonably practicable, into their proper (trade) names. Each of these proper names was given a unique code number (580 in total) and we then categorised these pesticides into whether they were OPs (and specifically diazinon) or non-OPs or could not be categorised.

Statistical analysis

Frequencies are presented for categorical data and means with standard deviations for continuous data. Odds ratios and *t*-tests were calculated to detect differences in categorical and continuous data, respectively. Logistic regression analysis was used to compute odds ratios and 95% confidence intervals. Adjustment for gender, age at first dipping and number of years of dipping had little effect on the odds ratios and hence unadjusted results are presented throughout.

The initial analyses included all cases and referents recruited for the study (Cherry et al. 2002, Mackness et al. 2003). Additional analyses were then carried out for populations known to be exposed to diazinon and after further refinement of the population. For those in each of the two exposure groups (392 without consideration of exposure and 255 exposed to diazinon), the populations under consideration were further refined by the symptoms or type of illness reported, following a plan of analysis described in more detailed elsewhere (Povey et al. 2005). In brief, at stage 1, 12 subjects with large numbers of missing data were excluded. At stage 2, 20 subjects were excluded as they had a chronic condition attributed by the general practitioner to a clearly defined event. At stage 3, this group together with 21 subjects with established neurological disease were excluded. At stage 4, subjects with established non-neurological disease were again excluded together with those whose current symptoms seemed, from a discriminant analysis, atypical of the case group. Finally, at stage 5, all subjects with chronic disease, both neurological and non-neurological, or with atypical symptoms, were excluded.

To detect an odds ratio of 2.0 with an $\alpha = 0.05$ and a power of 80%, the sample size of each arm of the study (cases and controls) needed to be between 145 and 200 assuming the prevalence of the polymorphism ranged between $\sim$15 and 45%. If the polymorphism prevalence was lower (between 5 and 10%), the power of the study would then be between 35 and 45%, assuming a population size (in each arm) of 200.

Results

Population characteristics

The study population has been described previously (Cherry et al. 2002, Mackness et al. 2003). Briefly, there were 409 subjects of which 175 were cases and 234 referents. A total of 156 (89%) and 210 (90%) of cases and referents were male (Table I). The majority of cases and referents, 112 (64%) and 155 (66%), respectively, lived in England and there was no difference in their geographical distribution. At interview cases were 2 years older than referents and they had first started dipping at

Table I. Comparison of cases and referents.

Variable	All ($n=409$)			Diazinon exposed ($n=267$)		
	Cases ($n=175$)	Referents ($n=234$)	p	Cases ($n=115$)	Referents ($n=152$)	p
Gender (% male)	89.1	89.7	0.48	93.0	90.1	0.27
Region (%)						0.50
Scotland/NI	13.7	12.0	0.18	12.2	13.2	
Wales	22.3	21.8		20.9	19.7	
North/NW England	15.4	11.5		13.0	9.9	
East/SE England	26.3	21.8		27.0	21.1	
SW England	22.3	32.9		27.0	36.2	
Age at interview (mean $\pm$ SD)	53.8 $\pm$ 10.5	51.8 $\pm$ 11.5	0.07	54.6 $\pm$ 9.1	50.7 $\pm$ 10.9	0.002
Year first dipped (mean $\pm$ SD)	1967.4 $\pm$ 12.3	1967.8 $\pm$ 12.6	0.77	1965.8 $\pm$ 12.0	1968.6 $\pm$ 11.9	0.06
Total years dipping (mean $\pm$ SD)	19.3 $\pm$ 7.6	22.0 $\pm$ 7.2	0.001	20.5 $\pm$ 7.0	22.0 $\pm$ 7.0	0.09

the same time but had spent significantly less time dipping than the referents (19.3 $\pm$ 7.6 versus 22.0 $\pm$ 7.2 years; $p < 0.001$).

Two-thirds of all participants reported that they had, at some time, used dips containing diazinon (115 (66%) cases, 152 (65%) referents). A total of 88 (22%) participants had not used diazinon but had used an organophosphate pesticide, and 54 (13%) could not give sufficient detail for classification. The majority of those who had used dips containing diazinon were men and again there was no difference in the geographical distribution of cases and referents. At interview, cases who had used diazinon, were 4 years older than referents who had used diazinon ($p = 0.002$), had started dipping earlier ($p = 0.06$) and had dipped for fewer years ($p = 0.09$).

PON1 genotype and phenotype

There was a significant difference in frequency of the *PON1-55* and *PON1-192* genotypes in cases and referents (Table II), both in the whole study population and in those cases and referents with some diazinon exposure. Risk associated with the *QR*/*RR* genotype did not vary with exposure to diazinon, whereas the risk associated with the *LL* genotype was somewhat higher in the diazinon-exposed population (OR 3.16, 95% CI 1.88–5.31) than in the whole population (OR 1.92, 95% CI 1.26–2.93). Diazoxonase activity was higher in the referents but not significantly so (Table II) but there was elevated risk associated with having low diazoxonase activity ($<$14.2 μmol min^{-1} ml^{-1} serum), which was not importantly greater in the diazinon-exposed population than in all subjects.

GST genotype

There were no significant differences in the frequency of the *GSTM1*2* (null) genotype and *GSTT1*2* (null) genotype in cases and referents in either the whole population or in only those who were exposed to diazinon (Table III). Restricting the population further by excluding subject groups 1–4 in the staged analysis also had little effect on these results (data not shown).

Table II. Association between *PON1-55*, *PON1-192* genotypes and PON1 phenotype (diazoxon hydrolysis) and self-reported ill health amongst sheep dippers.

Variable		Exposure	*n*	Cases	Referents	OR (95% CI)	*p*
PON 1-55 genotype	LL/LM/MM (%LL)	All	409	86/75/14 (49.1)	74/124/36 (31.6)	1.92 (1.26–2.93)[b]	–
		Diazinon	267	59/46/10 (51.3)	38/89/25 (25.0)	3.16 (1.88–5.31)[b]	–
PON 1-192 genotype	QQ/QR/RR (%QQ)	All	409	69/90/16 (39.4)	140/81/13 (59.8)	2.25 (1.49–3.42)[c]	–
		Diazinon	267	48/53/14 (41.7)	96/48/8 (63.2)	2.39 (1.45–3.93)[c]	–
Diazoxon hydrolysis	Mean ± SD (μmol min^{-1} ml^{-1} serum)	All	379	14.1 ± 5.3	15.0 ± 5.3	–	0.10
		Diazinon	247	14.2 ± 5.7	15.2 ± 4.8	–	0.12
Diazoxon hydrolysis	Low/high activity[a] (% low)	All	379	95/69 (57.9)	94/121 (43.7)	1.77 (1.15–2.73)[d]	–
		Diazinon	247	63/45 (58.3)	59/80 (42.5)	1.90 (1.11–3.27)[d]	–

[a]Based on median diazoxonase activity (14.2 μmol min^{-1} ml^{-1}).
[b]*LL* vs *LM+MM*.
[c]*QR+RR* vs *QQ*.
[d]Low vs high activity.

In the whole population, there was an elevated, but non-significant, risk associated with the combined *GSTM1* and *GSTT1* null genotype: 9% of cases, but only 5.8% of referents were double-null homozygotes (OR 1.61, 95% CI 0.74–3.48). In the staged analysis, there was evidence of an increased risk (Table IV). After exclusion of subjects on clinical grounds (analysis stage 3), 10.4% of cases were double-null homozygotes at stage 3 compared to 5.4% of referents: the odds ratio (95% CI) was 2.06 (0.85–5.04; Table IV). In the population restricted to those exposed to diazinon, the risk associated with being a double-null homozygote was slightly higher: at stage 3, 9.4% of cases were double-null homozygotes as compared to 3.9% of referents: the odds ratio (95%CI) was 2.60 (0.72–10.42; Table IV).

In the whole population, 35.6% of all cases, but 42.3% of referents, were *GSTP1*A* homozygotes; the risk associated with having a **B* or **C* allele was slightly increased, both in the whole study population (OR 1.33, 95% CI 0.87–2.01) and in those exposed to diazinon (OR 1.47, 95% CI 0.88–2.46; Table V). Risk of being a case varied little in staged analysis except in the population restricted to those exposed to

Table III. Association between *GSTM1* and G*STT1* genotypes and self-reported ill health amongst sheep dippers.

Gene	Genotype	Exposure	*n*	Case	Referent	OR (95% CI)
GSTM1	Present/deleted (% deleted)[a]	All	392	93/74 (44.3)	121/104 (46.2)	0.93 (0.62–1.38)[b]
		Diazinon	255	63/47 (42.7)	76/69 (47.5)	0.82 (0.48–1.40)[b]
GSTT1	Present/deleted (% deleted)[b]	All	392	135/32 (19.2)	185/40 (17.8)	1.10 (0.66–1.84)[b]
		Diazinon	255	89/21 (19.1)	121/24 (16.6)	1.19 (0.59–2.38)[b]

[a]Present = *GSTM1*1*; deleted = *GSTM1*2*.
[b]Present = GSTT1*1; deleted = GSTT1*2.
[b]Deleted (**2*) vs present (**1*).

Table IV. Associations between combined *GSTM1* and *GSTT1* genotype and case status.

	All population				Diazinon exposed			
		GSTM1/*GSTT1* genotype: both present/one deleted/both deleted (% both deleted)[a]				*GSTM1*/*GSTT1* genotype: both present/one deleted/both deleted (% both deleted)[a]		
Analysis stage	Total *n*	Case	Referent	OR (95% CI)[b]	Total *n*	Case	Referent	OR (95% CI)[b]
Full	392	76/76/15 (9.0)	95/117/13 (5.8)	1.61 (0.70–3.71)	255	51/50/9 (8.2)	60/78/7 (4.8)	1.76 (0.58–5.44)
1	380	72/74/15 (9.3)	92/115/12 (5.5)	1.77 (0.76–4.17)	247	49/49/9 (8.4)	57/77/6 (4.3)	2.05 (0.63–7.23)
2	360	67/71/15 (9.8)	84/112/11 (5.3)	1.94 (0.81–4.67)	236	46/49/9 (8.7)	52/75/5 (3.8)	2.41 (0.69–9.41)
3	339	57/63/14 (10.4)	83/111/11 (5.4)	2.06 (0.85–5.04)	215	36/41/8 (9.4)	51/74/5 (3.9)	2.60 (0.72–10.42)
4	301	52/49/12 (10.6)	75/102/11 (5.9)	1.91 (0.76–4.85)	191	27/32/7 (9.2)	45/65/5 (4.4)	2.23 (0.58–9.25)
5	293	45/43/12 (12.0)	78/104/11 (5.7)	2.26 (0.89–5.75)	186	31/28/7 (10.6)	47/68/5 (4.2)	2.73 (0.71–11.33)

[a]Both present = *GSTM1*1*/ *GSTT1*1*; one deleted = *GSTM1*2*/ *GSTT1*1 or GSTM1*1*/ *GSTT1*2*; both deleted = *GSTM1*2*/ *GSTT1*2*.
[b]Both deleted vs one deleted and both present.

Table V. Associations between *GSTP1* genotype and case status.

	All population				Diazinon exposed			
		*GSTP1 *A*A/ *A*B or *A*C/*B*B or *B*C or *C*C (% *A*A)*				*GSTP1 *A*A/ *A*B or *A*C/*B*B or *B*C or *C*C (% *A*A)*		
Analysis stage	Total *n*	Case	Referent	OR (95% CI)[a]	Total *n*	Case	Referent	OR (95% CI)[a]
Full	383	58/82/23 (35.5)	93/101/26 (42.2)	1.33 (0.88–2.01)	247	39/54/13 (36.8)	65/53/23 (46.1)	1.39 (0.63–3.09)
1	372	55/79/23 (35.0)	89/101/25 (41.4)	1.31 (0.84–2.05)	240	37/53/13 (35.9)	61/53/23 (44.5)	1.43 (0.82–2.51)
2	353	51/75/23 (34.2)	86/97/21 (42.2)	1.40 (0.88–2.22)	230	34/52/14 (34.0)	60/51/19 (46.2)	1.66 (0.94–2.96)
3	333	47/63/21 (35.9)	84/97/21 (41.6)	1.27 (0.79–2.05)	210	30/40/12 (36.6)	58/51/19 (45.3)	1.44 (0.78–2.64)
4	296	38/53/18 (34.9)	76/92/19 (40.6)	1.28 (0.76–2.15)	187	22/38/12 (30.6)	52/46/17 (45.2)	1.88 (0.96–3.67)
5	289	35/46/16 (36.1)	79/94/19 (41.1)	1.24 (0.73–2.12)	183	20/32/11 (31.8)	55/48/17 (45.8)	1.82 (0.92–3.63)

[a](*GSTP1 *A*B+GSTP1 *A*C+ GSTP 1*B*B+GSTP1 *B*C+GSTP 1*C*C*) vs *GSTP1 *A*A*.

diazinon. At stage 5, the risk associated with having a **B* or **C* allele was 1.82 (95% CI 0.92–3.63).

CYP genotype

A total of 5.9% of referents, but only 3.1% of cases, were *CYP2D6* homozygotes (defined using *CYP2D6*3* and *CYP2D6*4*). The risk associated with *CYP2D6 WT* was elevated but not to a significant extent (OR 1.47, 95% CI 0.83–2.60; Table VI). Restricting the population further by excluding subject groups 1–4 in the staged analysis also had little effect on these results (data not shown).

There were no significant differences in the distribution of *CYP3A4* and *3A5* genotypes between referents and cases. Subjects homozygous for *CYP3A4*1B* or *CYP3A5*3* were rare with only one of each identified in this population. The *CYP3A4 *A /*B* allele frequency did not differ between cases and referents, being 0.975/0.025 and 0.964/0.036, respectively. Similarly, the *CYP3A5 *1/*3* allele frequency did not vary, being 0.957/0.043 and 0.942/0.057 in cases and referents, respectively.

Combined PON1, GST and CYP genotypes

There was evidence that the risk associated with *PON1* polymorphisms may vary with *CYP2D6* genotype particularly in those exposed to diazinon (Table VII). The odds ratio (95% CI) associated with *QR/RR* polymorphisms was 5.75 (1.22–29.11) in subjects characterised as either *CYP2D6 HET* or *CYP2D6 HOM* but only 1.81 (0.99–3.38) in *CYP2D6 WT* subjects. The odds ratio (95%) associated with the *LL* genotype was 3.98 (2.08–7.68) and 1.11 (0.23–4.98) in subjects genotyped as *CYP2D6 WT* and *CYP2D6 HET/HOM*, respectively.

In strata defined by the *GSTP1* genotype, risk associated with *QR/RR* and *LL* genotype was slightly lower in the *A*A stratum than in the stratum that contained all other combinations (Table VII). The risk associated with *PON1-192 QR/ RR* or *PON1-55* LL polymorphisms did not vary in strata defined by *GSTT1 or GSTM1* genotype (data not shown) except that the odds ratio (95%) associated with the *LL* genotype was 4.08 (2.15–7.76) and 1.21 (0.29–5.16) in *GSTT1*1* and *GSTT1*2* subjects, respectively (data not shown).

Table VI. Association between *CYP2D6, CYP3A4* and *CYP3A5* genotypes and self-reported ill health amongst sheep dippers.

Gene	Genotype	Exposure	*n*	Case	Referent	OR (95% CI)
CYP2D6	*WT/HET/HOM*	All	378	138/16/5 (3.1)	179/27/13 (5.9)	1.47 (0.83–2.60)[b]
	(%HOM)[a]	Diazinon	247	89/12/3 (2.9)	114/21/8 (5.6)	1.51 (0.76–2.99)[b]
CYP3A4	**1A*1A/*1A*1B/ *1B*1B*	All	382	154/8/0 (4.9)	205/14/1 (6.8)	0.71 (0.29–1.72)[c]
	*(%*1A*1B+*1B*1B)*	Diazinon	247	101/6/0 (5.6)	130/10/0 (7.1)	0.77 (0.27–2.20)[c]
CYP3A5	**1*1/*1*3/*3*3*	All	388	148/12/1 (9.9)	201/26/0 (11.5)	0.68 (0.34–1.37)[d]
	*(%*1*3+*3*3)*	Diazinon	252	97/8/1 (8.5)	129/17/0 (11.6)	0.70 (0.30–1.65)[d]

[a]WT = no *2D6*3* or **4* allele; HET = 1 *2D6 *3* or **4* allele; HOM = 2 *2D6*3* or **4* or 1 **3* and **4* allele.
[b]WT vs HET+HOM.
[c](**1A*1B+*1B*1B*) vs **1A*1A*.
[d](**1*3+*3*3*) vs **1*1*.

Table VII. Associations between *PON1-55* and *PON1-192* genotype and diazoxonase activity and self-reported ill health amongst sheep dippers in strata of defined *CYP2D6* and *GSTP1* genotype.

			OR (95% CI)			
			CYP2D6[a]		*GSTP1*	
Variable	Exposure	Comparison	WT[a]	HOM/HET	*A*A	All other combinations
PON1-55	All	*LL* vs *LM/MM*	2.18 (1.35–3.47)	1.39 (0.47–4.11)	2.26 (1.09–4.66)	4.31 (1.69–11.17)
	Diazinon exposed		3.98 (2.08–7.68)	1.11 (0.23–4.98)	1.63 (0.90–2.96)	2.27 (1.06–4.87)
PON1-192	All	*QR/RR* vs *QQ*	1.93 (1.23–3.02)	3.30 (1.08–10.07)	1.37 (0.68–2.76)	2.22 (1.23–4.02)
	Diazinon exposed		1.81 (0.99–3.08)	5.75 (1.72–29.11)	1.80 (1.20–6.67)	2.68 (1.29–5.60)
Diazoxonase activity	All	Low vs high activity[b]	1.63 (1.00–2.69)	2.48 (0.69–9.07)	1.16 (0.56–2.40)	2.05 (1.14–3.70)
	Diazinon exposed		1.67 (0.90–3.10)	5.50 (1.03–36.84)	0.99 (0.40–2.40)	2.76 (1.28–5.98)

[a]WT = no *2D6*3* or **4* allele; HET = 1 *2D6 *3* or **4* allele; HOM = 2 *2D6*3* or **4* or 1 **3* and **4* allele.
[b]Based on median diazoxonase activity (14.2 μmol min^{-1} ml^{-1}).

Combined diazoxonase activity, CYP and GST genotypes

Risk associated with low diazoxonase activity was higher in those subjects genotyped as *CYP2D6 HET* or *CYP2D6 HOM* rather than *CYP2D6 WT*. In those exposed to diazinon, the odds ratio (95% CI) associated with low diazoxonase activity was 5.50 (1.03–36.84) in subjects characterised as either *CYP2D6 HET* or *CYP2D6 HOM,* but only 1.67 (0.90–3.10) in *CYP2D6 WT* subjects.

There was little evidence that risk associated with low diazoxonase activity varied with the *GSTT1* or *GSTM1* genotype (data not shown). In contrast, risk associated with low diazoxonase activity was higher in subjects with *GSTP1 *B* or **C* alleles, than in *GSTP1*A* homozygotes. In particular, in those exposed to diazinon the odds ratio (95% CI) associated with low diazoxonase activity was 2.76 (1.28–5.98) in subjects with *GSTP1*B* or **C* alleles but 0.99 (0.40–2.40) in *GSTP1*A* homozygotes (Table VII).

Discussion

Previously, we reported that self-reported chronic ill health amongst farmers exposed to organophosphates during sheep dipping was associated with *PON1* polymorphisms and PON1 phenotype and these results were consistent with the *a priori* hypothesis that OPs may be involved in the aetiology of ill health in this population (Cherry et al. 2002, Mackness et al. 2003). Furthermore, after the exclusion of subjects to provide a more homogenous case and referent population, the risk associated with *PON1* genotype varied little suggesting that the original analysis was robust (Povey et al. 2005). Associations between *PON1* gene polymorphisms and chronic symptoms in pesticide-exposed workers have since been reported, supporting this conclusion (Hernandez et al. 2003, Lee et al. 2003). PON1 is important in hydrolysing and inactivating OPs but it is likely that the risk of ill health following exposure will depend upon the balance between metabolic activation and metabolic deactivation and this balance will vary with the nature of the exposure. To further evaluate potential susceptibility factors in this population of sheep dippers, we have examined the distribution of common genotypes reportedly associated with chemical metabolism in the whole study population, in a more homogenous population after exclusion of subjects on clinical grounds and after discriminant analysis (Povey et al. 2005) and in those known to be exposed to diazinon. Our *a priori* hypotheses were that: (1) if risk associated with PON1 phenotype and *PON1* genotype varied with exposure, a more homogenously exposed population would show differences in risk more clearly; (2) individuals with increased metabolic activation and decreased non-PON1 detoxification pathways may be at increased risk; (3) risk associated with PON1 phenotype and *PON1* genotype may vary with other metabolic genotypes. Results suggest that indeed there may be altered risk associated with exposure and other metabolic genotypes.

Detailed assessment of the dippers' occupational history made it possible to specifically identify those who recalled being exposed to diazinon. The remaining subjects, who could not be so classified, could still have been exposed to diazinon but were more likely to have been exposed to other OPs or non-OP products. Hence, we evaluated the risk estimates for the original data in a population restricted to those known to be exposed to diazinon, as PON1 is known to be involved in the metabolism of diazinon. There was little evidence of a notable change in risk associated with the *PON1-192* genotype in this restricted population but the risk associated with the

LL PON1-55 genotype did increase (from an OR of 1.92 to 3.16) suggestive of a gene–environment interaction. The risk associated with the PON phenotype also did not vary but the PON1 phenotype measurement may be confounded by other factors such as disease status or co-existing exposures (Costa et al. 2003).

Previous studies have suggested that the CYP2D6 protein may play a role in activating diazinon to the toxic diazoxon (Sams et al. 2000). Increased metabolic activation by the protein, as would occur in *CYP2D6 WT*, would then presumably lead to increased risk. We thus carried out a restricted analysis of *CYP2D6* genotypes which would identify the majority of poor metabolizers in Caucasian populations (Smith et al. 1992, Bradford 2002). There was limited evidence of an association between the *CYP2D6 WT* genotype and self-reported ill health in that the odds ratio (1.47) was raised but not significantly. We then further examined whether previously reported associations between the PON1 phenotype and *PON1* genotype (Cherry et al. 2002, Mackness et al. 2003) varied depending upon *CYP2D6* genotypes. Given the putative metabolic role of the CYP2D6 protein, risk associated with the *CYP2D6 WT* strata would be anticipated to be greater than that with the *HET/HOM* strata. The risk associated with the *PON1-55 LL* genotype was increased in those with high CYP2D6 activity (*CYP2D6 WT* genotype) but increase risk associated with low diazoxonase activity and *PON1-192 QR/RR* genotype was observed in subjects with reduced and not increased CYP2D6 activity (i.e. *CYP2D6 HET/HOM* genotype). These results are thus not fully consistent with our initial predictions of an increased risk associated with high CYP2D6 activity. The precise significance of these results is unclear, and may indeed have occurred by chance or by misclassification of the CYP2D6 phenotype, as we have not identified certain genotypes (Zanger et al. 2004).

There was no evidence of a direct association between *CYP3A4* and *3A5* polymorphisms and self-reported chronic ill health. This lack of an association may simply reflect the low prevalence of the examined polymorphisms in this study population. Their low prevalence does rule out a major effect of these polymorphisms, at least in UK populations, but does not necessarily rule out any phenotypic associations between 3A4 and 3A5 activity and ill health.

There was some evidence of an association with *GSTP1*B* and *GSTP1*C* polymorphisms and self-reported chronic ill health irrespective of *PON-1* genotype and phenotype. *GSTP1*B* and *GSTP1*C* alleles have been reported to alter the specificity to certain substrates, and patients with Parkinson's disease who reported exposure to pesticides were more likely to have *GSTP1* non-A alleles than referents (Menegon et al. 1998). If true, the risk associated with the *GSTP1*B* and *GSTP1*C* alleles would suggest that the metabolism of OPs by *GSTP1*B* and *GSTP1*C* alleles would be quantitatively or qualitatively different *in vivo* to that afforded by the **A* allele and such a hypothesis could be readily examined *in vitro.* There was little evidence of associations between *GSTM1* and *GSTT1* polymorphisms and risk of being a case, although risk associated with the *PON1-55 LL* genotype may vary with strata defined by the *GSTT1* genotype. The lack of an association between the null *GSTM1* and *GSTT1* genotype and case status may simply reflect the unimportance of these metabolic pathways in removing toxic OP metabolites (in contrast to the well-recognised PON1 pathway). Alternatively, it has been suggested that redundancy in these metabolic pathways may obscure any associations between specific *GST* genotypes and disease risk (Helzlsouer et al. 1998), and indeed there was some evidence of an effect associated with double-null (*GSTM1*, *GSTT1*) homozygotes

with a doubling of risk. The suggestive increase in risk association with the *PON1-55 LL* genotype and the *GSTT1*1* may simply be a chance finding as, *a priori*, the risk would have been expected to decrease with the presence of GSTT1, although GSTT1 has been associated with the metabolic activation rather then detoxification of certain substrates (Thier et al. 1996).

Hence, these results suggest that there may be additional differences in metabolic genotypes between cases and referents in this population but the differences in risk were neither strong nor entirely consistent with *a priori* hypotheses of risk associated with particular genotypes. This may be due to the relative lack of knowledge of the role of PON1 isoforms and other proteins in metabolising specific organophosphates and indeed other chemicals. Further work is required to define more clearly the metabolic pathways of specific organophosphates (Fabrizi et al. 1999, Mutch et al. 1999, Kappers et al. 2001, Sams et al. 2004) and the precise nature of the toxic metabolite(s) involved. For example, CYP2C19 has recently been implicated in diazinon metabolism (Kappers et al. 2001) but the low prevalence of CYP2C19-poor metabolizers in Caucasian populations (Mizutani 2003) suggests that this protein could not play an important role in determining genetic susceptibility to diazinon toxicity. These results suggest that other non-PON1 pathways may be implicated – a result which can be readily tested using *in vitro* assays.

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