

Pharmacogenetic Profile of Xenobiotic Enzyme Metabolism in Survivors of the Spanish Toxic Oil Syndrome

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In 1981, the Spanish toxic oil syndrome (TOS) affected more than 20,000 people, and over 300 deaths were registered. Assessment of genetic polymorphisms on xenobiotic metabolism would indicate the potential metabolic capacity of the victims at the time of the disaster. Thus, impaired metabolic pathways may have contributed to the clearance of the toxicant(s) leading to a low detoxification or accumulation of toxic metabolites contributing to the disease. We conducted a matched case-control study using 72 cases (54 females, 18 males) registered in the Official Census of Affected Patients maintained by the Spanish government. Controls were nonaffected siblings ($n=72$) living in the same household in 1981 and nonaffected nonrelatives ($n=70$) living in the neighborhood at that time, with no ties to TOS. Genotype analyses were performed to assess the metabolic capacity of phase I [cytochrome P450 1A1 (CYP1A1), CYP2D6] and phase II [arylamine *N*-acetyltransferase-2 (NAT2), GSTM1 (glutathione *S*-transferase M1) and GSTT1] enzyme polymorphisms. The degree of association of the five metabolic pathways was estimated by calculating their odds ratios (ORs) using conditional logistic regression analysis. In the final model, cases compared with siblings (72 pairs) showed no differences either in CYP2D6 or CYP1A1 polymorphisms, or in conjugation enzyme polymorphisms, whereas cases compared with the unrelated controls (70 pairs) showed an increase in NAT2 defective alleles [OR = 6.96, 95% confidence interval (CI), 1.46–33.20] adjusted by age and sex. Glutathione transferase genetic polymorphisms (GSTM1, GSTT1) showed no association with cases compared with their siblings or unrelated controls. These findings suggest a possible role of impaired acetylation mediating susceptibility in TOS. **Key words:** CYP1A1, CYP2D6, enzyme genetic polymorphisms, GSTM1, GSTT1, molecular epidemiology, NAT2, Spanish toxic oil syndrome, xenobiotic metabolism. *Environ Health Perspect* 109:369–375 (2001). [Online 16 March 2001] <http://ehpnet1.niehs.nih.gov/docs/2001/109p369-375ladona/abstract.html>

Among food-related toxic outbreaks that have occurred in the world, the Spanish toxic oil syndrome (TOS) emerges as a significant disaster because of the degree of severity and the huge population involved (1,2). In May 1981 the TOS appeared in Madrid and northwestern areas of Spain as a unique disease caused by the ingestion of adulterated rapeseed oil denatured with aniline (3–7). More than 20,000 people were affected; of these, over 11,000 required hospitalization and over 300 deaths were registered in the first 2 years (1,8). Although the majority of patients recovered after a long period, 30–40% continue to suffer mild symptoms or severe sequelae (9–12). TOS was characterized as a multisystemic disease with three consecutive phases. In the acute phase (1–2 months), patients presented fever, rash, eosinophilia, pulmonary edema, and myalgia. Many patients (59%) progressed to an intermediate phase with pulmonary hypertension, thromboembolism, persistent myalgia and eosinophilia, skin edema, alopecia, and sicca syndrome. The clinical signs of the chronic phase were principally pulmonary hypertension, scleroderma, peripheral neuropathy, and liver disease. A summary of clinical and

epidemiological findings has been compiled in recent reviews (9–11,13,14).

Rapeseed oil, denatured with 2% aniline, was imported for industrial purposes and illegally refined and delivered for human consumption. A strong association of TOS with ingestion of this oil was proven (3–5,15); thus, the syndrome was caused by toxicants in the oil (1,6,7,13,15,16). Despite the analytical efforts seeking toxic substances in these oils, only aniline derivatives such as fatty acid anilides (1,3,17,18) and fatty acid esters of 3-phenylamino-1,2-propanediol (PAP esters) (19–21) have been identified in toxic oil batches. The content of oleanilides and PAP esters in the oil has been strongly associated with the morbidity caused by these oil batches in the corresponding households (16,22,23). In particular, the di-oleyl-PAP ester (OOPAP) is considered the putative toxic substance generated during the refining process (23,24); however, its toxicity mechanism in biological systems has not yet been fully clarified. Extensive experiments in diverse animal species fed with toxic oil or administered aniline derivatives have failed to reproduce the full spectrum of the disease (1,25,26). This may suggest a species-specific

toxicity for humans; in this respect, species differences in aniline toxicity have been recognized for decades and attributed to metabolism differences (27–30).

The disease tended to cluster in families, and the exposure factor was shown to be closely related to household life (4–7). Nevertheless, members of the same family seemed to differ in their risk of becoming ill (4,5), which suggested consumption of different amounts of the oil (a dose factor) and/or a susceptibility trait. With respect to the latter, an immunological mechanism was initially suggested as a toxicity target and was extensively investigated (31–33) because the disease resembled an allergic-toxic syndrome in the acute phase and an autoimmune condition in the chronic phase. However, with regard to a dose factor in toxicity, the patients' detoxification mechanisms have not yet been investigated. The real toxic dose ingested by patients before the oil was officially recalled was unknown. Epidemiological studies on dietary habits in 1981 failed to conclusively establish a correlation between oil consumption and severity of the disease (4,5). However, these studies did not provide analytical data on aniline-derivative content in the household-distributed oil batches; to date, it is known that oleylanilide and OOPAP content varied several folds in oil batches (18,22–24). This would suggest that some families might have suffered a poisoning dose due to a high toxicant(s) content in their edible oil batch, whereas other families may have reflected a susceptible trait

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even with low toxicant content in their oil batch (Figure 1).

TOS, as the result of a toxic chemical ingestion, would invite investigation on the subject's capacity to biotransform and eliminate the toxic agent(s). Thus, differences in xenobiotic metabolism and inherited genes among exposed subjects may have contributed to the overall clearance and elimination of the toxicant(s), resulting in an accumulation of toxic metabolites, or a low detoxification, contributing to the disease. In this context, polymorphic genes that encode drug-metabolizing enzymes are attractive candidates for unraveling mechanisms of genetic susceptibility in adverse drug reactions or in xenobiotic exposure toxicity (34,35). Phase I enzymes may metabolically activate xenobiotics and procarcinogens, yielding toxic or carcinogenic electrophiles, respectively; phase II enzymes may be implicated in detoxifying such products. In this study we attempt to identify host-metabolism differences (i.e., genetic susceptibility factors) that may have played a role in the pathogenesis of TOS. In other words, our goal was to determine whether the TOS population inherited a particular genetic profile with regard to xenobiotic enzyme metabolism, which would imply impaired or increased metabolic capacity toward chemical exposure.

Methods

Population selection and study variables. The study was designed as a matched case-control study with two different controls—nonaffected siblings and unrelated nonaffected subjects—hereinafter referred to as siblings and friends, respectively. Cases were recruited from five areas where the Associations of TOS-Affected Patients cooperated.

Inclusion criteria for cases were the same as those used in other TOS studies (16); cases included patients registered in the TOS Official Census who underwent an acute and/or chronic phase of the disease. The acute phase is defined as an alveolo-interstitial lung infiltration and/or pleural effusion with absolute eosinophilia (> 500 cells/mm³). The chronic phase is defined as myalgia and eosinophilia, and/or scleroderma, neuropathy, pulmonary hypertension or hepatopathy clearly attributed to TOS disease. Siblings were selected from among brothers or sisters who lived with the case and shared the same meals with him/her when the epidemic started in 1981 and when the case became ill. After we selected the case and his/her sibling, the case himself/herself chose the unrelated control from among his/her friends, provided the friend had lived in the same locality in 1981 when the epidemic broke out and had had no affected family members. Exclusion criteria included pregnancy, age > 65 years, mental disorders, and reluctance to collaborate in the study. Neither siblings nor friends had to present symptoms or signs of the illness. We gave a questionnaire and an informed consent form to each selected person. All subjects recruited for this study were informed of the aims of the investigation in detail and asked to give their written consent to participate. The study was approved by the Ethics Committee of Hospital 12 de Octubre (Madrid).

Recruited subjects were coded by random numbers; blood samples were collected, correspondingly labeled, and frozen at -80°C until analysis. All analytical determinations and questionnaire data management were carried out by personnel unaware of the identity or biochemical data of the patient. Two

variables described case or control status and its specific matching group. Other variables were geographical residence in 1981, age, sex, and health status. Metabolic variables to be studied were genetic polymorphisms in xenobiotic metabolism phase I [cytochrome P450 1A1 (CYP1A1), CYP2D6] and phase II [arylamine *N*-acetyltransferase-2 (NAT2), glutathione *S*-transferase M1 (GSTM1) and GSTT1] enzymes.

Genotyping analysis. We analyzed all of the samples in blind conditions with regard to case-control status. Genotype analyses were made on genomic DNA isolated from blood collected in EDTA tubes and frozen at -80°C until assayed. Genomic DNA was extracted from the leukocyte pellet by standard phenol extraction followed by isopropanol precipitation and was stored at 4°C in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Genotypes were assessed by polymerase chain reaction (PCR) allele-specific amplification of functional genes. Further nested PCR or enzyme restriction endonuclease (PCR-RFLP) analyses permitted assessment of specific point mutations in intron-exon sequences known to impair enzyme activity. We determined the *CYP2D6* gene locus by genomic RFLPs using a non-radioactive Southern blot technique and following the manufacturer's instructions (DIG DNA labeling and DIG luminescent detection kits; Boehringer Mannheim, Germany). We established specificity of the PCR techniques and confirmed PCR products by sequencing. Homozygous and heterozygous control samples were subsequently included in all reactions. All samples with mutations and 10% of samples with wild type/wild type (*wt/wt*) haplotype were confirmed. We used two molecular methods to assess mutations in NAT2 and CYP2D6 polymorphisms, thereby confirming results. Therefore, these quality control measures fully validated the participants' genotypes.

Methods to determine CYP2D6 point mutations (A₂₆₃₇ deletion, G_{1934A}) were established as described elsewhere (36,37). Gene deletions and duplications were identified by *Xba*I/*Eco*RI RFLPs (38) using a cDNA probe provided by U.A. Meyer (Biocentre, Basel, Switzerland). Combined PCR and RFLPs analyses defined CYP2D6 genotypes according to established nomenclature (39). These genotypes are believed to account for 95% of the known CYP2D6 polymorphism (40–42). With regard to CYP1A1 polymorphisms, two *Msp*I sites reported in ethnic differences were detected by PCR-RFLPs described at the 3' gene-flanking region, and a point mutation in exon 7 at codon 462 (A_{4889G}) producing an amino acid exchange (isoleucine-valine) was also determined (43–45). We used the

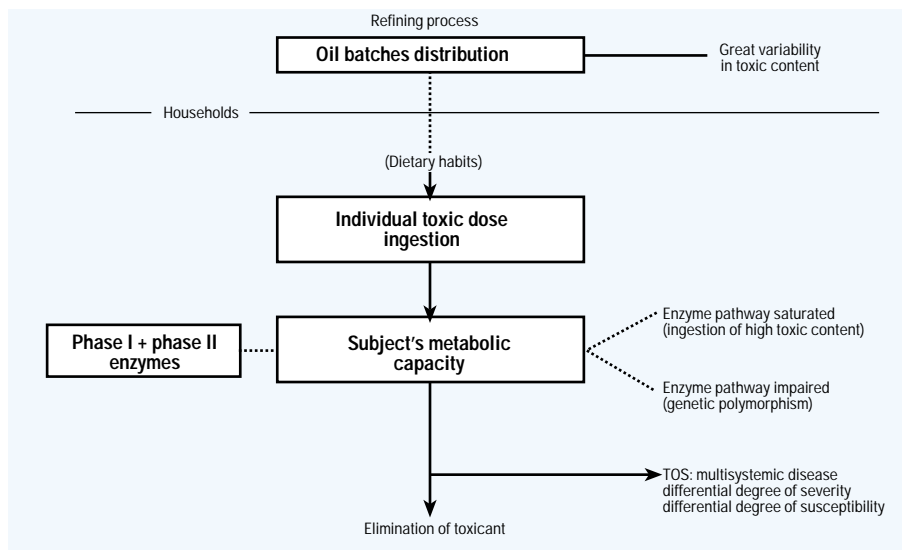


Figure 1. Factors that contribute to TOS differential morbidity observed in households: toxic dose and metabolism.

nomenclature of Taioli et al. (45) to name the *CYP1A1* genotypes: C, wild-type allele; M, the allele with the *MspI* site at the 3′-flanking region (T₆₂₃₅C); D, the allele with *MspI* site plus valine mutation; and A, the allele with the *MspI* site at 5315 nucleotide (T₅₃₁₅C).

Gene deletions causing impairment of *GSTM1* (46) and *GSTT1* (47) were analyzed by well-described allele-specific PCR methods (47,48). These methods permitted identification of the homozygous deleted gene, the so-called null allele (*GSTM1*− or *GSTT1*−), from the heterozygous and homozygous wild type haplotype, the *wt* allele (*GSTM1*+ or *GSTT1*+). Finally, we assessed the NAT2 genetic polymorphism to identify *m1* (T₃₄₁C, C₄₈₁T), *m2* (G₅₉₀A), and *m3* (G₈₅₇A) point mutations in the coding region (49). These mutations account for 90–95% of the enzyme’s capacity variability described for NAT2 polymorphism (41,50,51).

All chemical reagents, of molecular and analytical grade, were purchased from Sigma Chemical (St. Louis, MO, USA) and Merck (Darmstadt, Germany). We obtained synthetic primers and 2′-deoxynucleosides-5′-triphosphate from Pharmacia Biotech (Uppsala, Sweden), restriction enzymes from Gibco-BRL (Gaithersburg, MD, USA), and

Taq polymerase from Perkin Elmer (Norwalk, CT, USA).

Statistical analysis. Each genetic variable was tested separately for its distribution in each group (cases, siblings, and friends) and given as allele frequencies.

We defined three metabolic categories on the basis of their functional and nonfunctional derived haplotypes: *wt/wt*, *wt/mutant* (*wt/m*), and *m/m*. We then designed dummy variables to evaluate the independence of each genetic category. The absence of mutations (*wt/wt*) was considered the metabolic basal risk, which was compared with the heterozygous (*wt/m*) or homozygous (*m/m*) mutations. We used univariate conditional logistic regression to measure the relative risk of cases versus siblings and cases versus friends. Data are shown as odd ratios (ORs) with 95% confidence intervals (CIs).

We performed multivariate analysis by applying multivariate conditional logistic regression following a backward strategy, including all metabolic pathways of phase I and phase II to test their interactions and adjusting by age and sex. Variables were retained if they achieved statistical significance ($p \leq 0.05$) or if, in order to control confounders, their absence changed the remaining estimated coefficients by at least 15%. To give adjusted estimators, sex and age were also retained despite their significance. We used the change in the $-2 \log$ likelihood to compare different models. Analyses were performed with the Epidemiological Graphics Estimation and Testing software (EGRET, analysis module version 0.26.6, EPIXACT version 0.03 1985–1991; Cytel, Cambridge, MA, USA).

Results

We collected and analyzed samples from 236 subjects (80 cases, 80 siblings, and 76 friends) with their corresponding questionnaires. We excluded 21 subjects from the statistical analyses. Of these, 7 cases were excluded because 2 were not officially registered as cases and 5 did not fulfill inclusion criteria; their corresponding 7 siblings were also excluded. In addition, 1 sibling was

excluded because there was no age match (born after the outbreak). In the friends group, 4 were excluded because they had no case for comparison and 2 were excluded because they did not live in same geographical area as their case at the time of the disaster. As a result, we identified 215 subjects as 73 cases, 72 siblings, and 70 friends, thus yielding 72 and 70 matched-pair series for comparison. Women were overrepresented among cases (75% vs. 54% in siblings and 48.6% in friends). On average, cases were younger than their friends but not younger than their siblings (median: 27 years for cases, 26.5 years for siblings, and 29 years for friends). The clinical symptom profile of the included cases at the time of the outbreak is shown in Table 1.

Descriptive results of phase I metabolic pathways, *CYP1A1* and *CYP2D6* enzyme genetic polymorphisms are shown in Table 2 and Figure 2. Allele frequencies of *CYP1A1* polymorphism in the seventh exon (isoleucine/valine amino acid exchange) and at the 3′ gene-flanking region (*MspI* restriction sites) are listed in Table 2. Cases presented a higher frequency of mutated alleles with *MspI* and valine point mutations (*M*, *A*, and *D* alleles, respectively); however, they did not reach statistical significance compared to controls. Mutation at the seventh exon was always linked to the presence of the 3′ *MspI* site (T₆₂₃₅C), i.e., the *D* allele. For two subjects in the friends group, the *MspI* restriction site reported for African Americans (44) was confirmed by sequencing analyses. The functional metabolic categories established included the haplotype combinations of *wt* (*C* allele) and the mutant alleles (*M* or *D*) (Figure 2A) and reflected a higher proportion of *CD* haplotype in the case group compared to friends (OR = 2.8; 95% CI, 0.9–9.0).

CYP2D6 metabolic capacity was assessed by determining point mutations and the genomic locus, as described in “Methods.” Genotype categories were defined according to established nomenclature (39). *XbaI* and *EcoRI* restriction endonucleases permitted detection of deletions and clear differentiation of 44 and 42 kb fragments. No subject

Table 1. Characteristics of TOS cases ($n = 72$).^a

Clinical features in 1981	<i>n</i>	%
Eosinophilia	66	91.7
Pulmonary disease	64	88.9
Myalgia	66	91.6
Neuropathy	23	31.9
Scleroderma	20	27.8
Hepatopathy	6	8.3
Pulmonary hypertension	4	5.6
Eosinophilia + myalgia	60	83.3
Eosinophilia + pulmonary + myalgia	53	73.6
Eosinophilia + myalgia + neuropathy	22	30.6

^aWe used the same inclusion criteria for cases as used previously (16).

Table 2. Phase I metabolism: allele frequencies of *CYP1A1* and *CYP2D6* genetic polymorphisms.

Allele ¹	Cases ($n = 73$)	Siblings ($n = 72$)	Friends ($n = 70$)
<i>CYP1A1</i>			
C	0.86	0.89	0.90
A	–	0.01	0.01
M	0.06	0.05	0.06
D	0.08	0.05	0.03
<i>wt</i>	0.86	0.89	0.90
<i>m</i>	0.14	0.11	0.10
<i>CYP2D6</i>			
1*	0.66	0.72	0.73
2 × 2*	0.06	0.05	0.03
3*	0.02	0.03	–
4*	0.25	0.20	0.21
5*	0.01	–	0.03
<i>wt</i>	0.72	0.77	0.76
<i>m</i>	0.28	0.23	0.24

Haplotypes (2 × 2* mutation or deletion) were categorized *wt/wt* as having 2 functional genes, i.e., *CYP2D6* 2 × 2*. Nomenclature is as reported for *CYP1A1* (46) and *CYP2D6* (39).

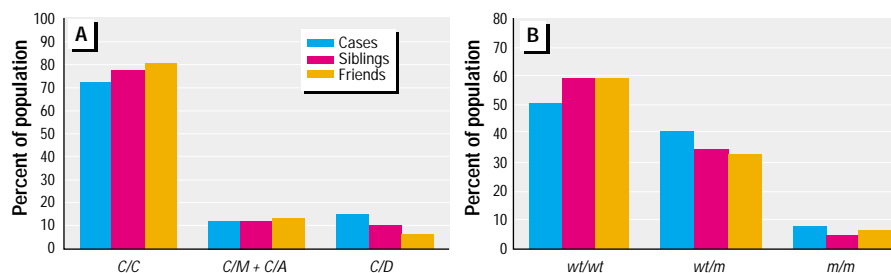


Figure 2. Phase I metabolism of *CYP1A1* and *CYP2D6* genetic polymorphisms. (A) *CYP1A1* functional haplotype categories given as a percentage. (B) *CYP2D6* functional haplotype categories given as percentages. *wt* indicates a functional allele, and *m* indicates a nonfunctional allele.

presented homozygous gene deletions (i.e., the 11.5 kb fragment). The *Xba*I 16+9 fragments were linked to a G1934A mutation and accounted for 3% in the entire sample collection. The *Xba*I-derived and *Eco*RI-confirmed 44 kb fragment was also linked to the presence of the splicing defect G₁₉₃₄A, as determined by PCR analyses. In contrast, the duplication fragment i.e., the 42 kb allele was always associated with *wt* PCR alleles and was clearly differentiated from 44 kb fragments by *Eco*RI restriction endonuclease. A novel *Xba*I RFLP of 33+9 kb was found in three subjects as a heterozygous 29 kb haplotype with an *Eco*RI pattern indistinguishable from the 42 kb allele. Thus, this may suggest that a new *Xba*I mutation had appeared in a 42 kb allele, giving the 9 kb fragment (52). This 33+9 RFLP fragment was also linked to mutation G₁₉₃₄A and considered as a *CYP2D6**4 nonfunctional allele. Prevalence of *CYP2D6* defective alleles among cases was higher compared to their relative controls (Figure 2B), which could imply that an impaired metabolism may have operated in some patients.

Genotype distributions of conjugation pathways (glutathione *S*-transferase and arylamine-*N*-acetyl-transferase major detoxification pathways) are shown in Table 3 and Figure 3. NAT2 point mutations (*m1*: C₃₄₁, T₄₈₁; *m2*: A₅₉₀; *m3*: A₈₅₇) causing enzyme-impaired function by posttranscriptional mechanisms were distributed in eight

Table 3. Phase II metabolism: allele frequencies of NAT2 and GST genetic polymorphisms.

Allele ¹	Cases (<i>n</i> = 73)	Siblings (<i>n</i> = 72)	Friends (<i>n</i> = 70)
NAT2			
<i>wt</i>	0.27	0.30	0.38
<i>m1</i>	0.38	0.43	0.37
<i>m2</i>	0.32	0.25	0.23
<i>m3</i>	0.03	0.02	0.02
<i>m</i>	0.73	0.70	0.62
GSTM1			
<i>GSTM1+</i>	0.52	0.54	0.46
<i>GSTM1-</i>	0.48	0.46	0.54
GSTT1			
<i>GSTT1+</i>	0.71	0.74	0.69
<i>GSTT1-</i>	0.29	0.26	0.31

Nomenclature is as reported for NAT2 (49), GSTM1 (48), and GSTT1 (47).

different haplotype combinations with the *wt* functional allele. Allele frequencies are listed in Table 3. A higher prevalence of defective alleles, in particular the *m2* and *m3* allele frequencies, was observed in cases compared with friends. The sibling group presented a higher prevalence of the *m1* allele than the friend controls. The metabolic categories derived from haplotype combinations reflected a lower proportion of functional haplotypes (*wt/wt*) among cases compared with friends (Figure 3A), thus suggesting that some impaired NAT2 function may be associated with TOS cases.

Gene deletions in GSTM1 and GSTT1 isoenzymes were determined by PCR (Table 3). Gene prevalence of homozygous null haplotype in GSTM1 and GSTT1 was almost equally distributed among groups (Figure 3B,C) and was also equally distributed after age and sex stratification. In considering genetic combinations of both enzymes, we observed two subgroups of cases: patients with a null haplotype in both GST enzymes (18% with *GSTM1-/GSTT1-*) or patients with a *wt* allele for both enzymes (41% with *GSTM1+/GSTT1+*). In the present study, it is impossible to establish whether this observation indicates subpopulations with variability in their clinical presentations, characteristics, or phenotype.

We performed statistical analyses to test the hypothesis that case subjects may present a different metabolic profile from their controls, which would indicate that the enzyme metabolic capacity of case subjects was associated with and challenged by TOS xenobiotic intoxication. Instead of comparing the allele frequencies observed, we chose to study genetic functional capacity because enzyme activity is determined by the presence and/or absence of mutations. Moreover, enzymatic capacity of haplotypes with one or both functional alleles would be subsequently challenged if doses were accumulative. Thus, homozygous mutant haplotypes would be the first at risk, and heterozygous and homozygous wild types would consecutively be involved because increasing doses would exhaust the subject's metabolic capacity. Using the three genetic categories (*wt/wt*,

wt/m, and *m/m*) as dummy variables, we evaluated independently the risk in CYP1A1, CYP2D6, NAT2, GSTT1, and GSTM1 enzymatic pathways. Table 4 shows the percentage distribution of all metabolic variables considered among the two matching series (cases vs. siblings, cases vs. controls). As stated above, we contrasted *wt/wt* haplotypes with the sum of heterozygous and homozygous mutated haplotypes.

The degree of association for each variable was assessed by conditional logistic regression analysis and the odds ratio of each was calculated. The estimated odd ratios in the final conditional logistic regression model adjusted by all metabolic variables, sex, and age, as described in "Methods," are shown in Table 5. In the final models, we excluded enzyme polymorphisms that failed to add any information to the model. We observed no differences in phase I metabolism among cases compared with their corresponding siblings or in the conjugation enzymes tested. In contrast, comparison with friends showed a distinct metabolic profile in cases with a high prevalence of defective *NAT2* alleles (*wt/m* + *m/m*) (OR = 6.96; 95% CI, 1.4–33.2).

Discussion

It is difficult and challenging to assess metabolic profiles that occurred 19 years ago at the time of the massive intoxication. Epigenetic modulation of genetic load expression in surviving patients caused by TOS disease or physiological and environmental factors should have operated in each subject's present metabolic capacity (present phenotype); however, each subject's pharmacogenetic profile would have been the same. Therefore, pharmacogenetic determinations made now may yield interesting information for explaining the subjects' interindividual differences in susceptibility to past toxicant exposure and would provide better understanding of the detoxification/toxication mechanisms involved in massive toxic outbreaks. After years of research on TOS, this study is the first to point out a specific metabolic profile in patients, that is, an increase in *NAT2* defective alleles. Thus, patients with *NAT2*

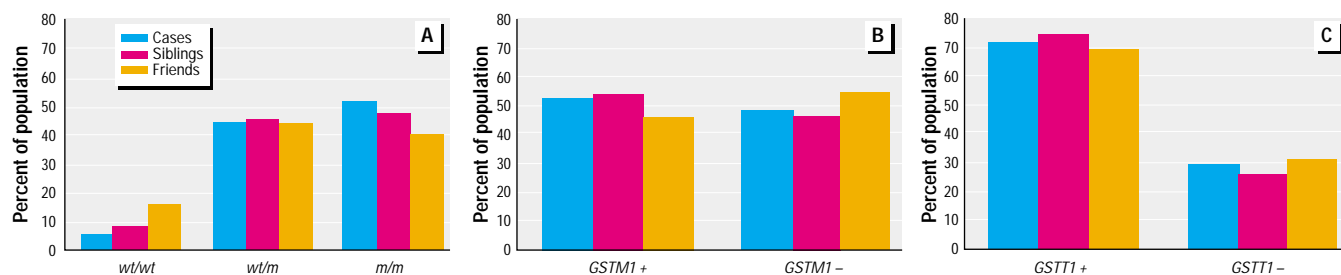


Figure 3. Phase II metabolism: conjugation pathways of NAT2 and GST genetic polymorphisms. Functional categories of (A) NAT2, (B) GSTM1, and (C) GSTT1 expressed as a percentage in the population.

homozygous mutant haplotypes would be the first at risk; heterozygous and homozygous wild types consecutively would be involved because accumulative doses would exhaust each subject's metabolic capacity. Some factors should be highlighted in connection with the TOS problem: *a*) a large proportion of the population was exposed compared with the number of cases; *b*) patients presented a great diversity of clinical manifestations of the disease; and *c*) a different degree of morbidity was observed among members of the same household who shared meals. These aspects suggest that a metabolic factor is the basis of these differences. Genetic, immunologic, or metabolic factors are frequently involved in the pathogenesis of this type of disease (53).

Alternatively, the present results may point to a metabolic characteristic of TOS survivors with regard to TOS deceased patients. Thus, a particular metabolic profile of the survivors of TOS may have acted as a prognostic factor more than as a risk factor. It was impossible to confirm this hypothesis, which would have substantially reinforced our results, because of the absence of frozen tissue samples at the beginning of the study. We attempted to extract DNA from tissue blocks fixed in formalin and were unsuccessful due

to the quality of the available samples at the time of the study.

One of the best designs for investigating potential risk factors in an outbreak is a case-control study (54). With regard to the control group, the selection of "the best friends of the case" has been used successfully (55). Moreover, we selected two different control groups, siblings and friends, to test the hypothesis under different scenarios. Both controls groups were used only on the basis of the absence of TOS disease, but not as representative of a population (no sampling was performed). Nevertheless, *CYP2D6* and *NAT2* allele frequencies found in friends were similar to those reported in other studies with larger Spanish populations (56–58). To avoid confounding factors, we decided to choose matched controls to adjust for exposure to oil consumption and other habits. However, one of the drawbacks of this kind of design is the possibility of overmatching (55,59). In our opinion, this may help to explain the absence of significant *NAT2* results in cases/siblings comparison. We assumed that siblings and cases had experienced the same exposure, although slight differences in the amount of oil ingested could have influenced results. In contrast, we were not able to check whether the group of

friends actually were nonexposed. Chemical analysis of the oils collected from the epidemic showed that more toxic oil was sold than was consumed by the families with cases (60). Thus, we can assume that unaffected subjects and cases from the same population area had the same probability of being exposed.

It is impossible to recognize any individual biomarker before analyses are performed unless the marker and the case selection methods are associated. Being related to a specific group of victims or being a friend of a case cannot justify a selection bias for this specific metabolic profile. Thus, the pharmacogenetic differences observed among these groups could only be explained by a true risk factor. It was not feasible to sex-match the group of siblings. Similarly, we also decided not to pair the friends group by sex; in fact, none of the genetic factors under study are sex-linked. Thus, the presence of defective alleles in *NAT2* and female cases in the final logistic regression models cannot be justified by intergroup sex differences. The overrepresentation of females among cases was a feature of the TOS epidemic (1,4,5). One explanation may be a chance exposure related to household life or an unidentified factor, perhaps epigenetic modulation, associated with females.

An interesting point regarding xenobiotic metabolism being a susceptibility factor is that some pathways are tissue-specific markers. It is feasible that the toxic agent(s) in the oil would have followed two possible absorption routes: directly to the lung through the thoracic duct or through the liver (3). Ultimate toxic derivatives in the blood stream may therefore be the result of these metabolic circuits. Moreover, experiments in rabbits and mice known to have *NAT* polymorphisms, revealed toxication symptoms depending on the administration route (25,61). Anilides and PAP esters identified in oil batches should be considered arylamides and arylamines, respectively. These two types of chemical species differ in their basicity, nucleophilicity, and ionization potential; as a consequence, their chemical reactivity and biotransformation may follow different pathways, resulting in several nucleophiles (62). In addition, the fatty acid moiety in either anilides or PAP esters confers a lipophilic characteristic for their distribution. These compounds share a chemical characteristic at the aromatic moiety of being oxidized; this is followed by complex conjugations, reductions, and/or hydrolysis (62), plausibly by some of these enzyme pathways reported in the present study. In particular, the contribution of *CYP1A* and *NAT2* enzymes to the metabolism of aniline-derived xenobiotics such as acetanilide and phenacetin is well known (63). In this respect, it is worth noting

Table 4. Metabolic study variables among matching pairs of cases/siblings ($n = 72$) and cases/friends ($n = 70$).

Variable	Case/sibling pairs		Case/friend pairs	
	Cases No. (%)	Siblings No. (%)	Cases No. (%)	Friends No. (%)
Sex				
Male	18 (25)	33 (45.8)	18 (25.7)	36 (51.4)
Female	54 (75)	39 (54.1)	52 (74.3)	34 (48.6)
<i>CYP1A1</i>				
(<i>C/D</i>) ^a	11 (15.3)	7 (9.7)	11 (15.7)	4 (5.7)
(<i>C/D + C/M,A</i>) ^a	20 (27.8)	16 (22.2)	20 (28.6)	13 (18.6)
<i>CYP2D6</i> (<i>wt/m + m/m</i>) ^b	34 (47.2)	29 (40.3)	33 (47.1)	28 (40)
<i>NAT2</i> (<i>wt/m + m/m</i>) ^b	68 (94.4)	66 (91.6)	66 (94.3)	59 (84.3)
<i>GSTM1</i> (<i>GSTM1</i> -) ^c	35 (48.6)	33 (45.8)	33 (47.1)	38 (54.3)
<i>GSTT1</i> (<i>GSTT1</i> -) ^c	21 (29.1)	19 (26.4)	20 (28.6)	22 (31.4)

Distribution of functional metabolic categories derived from Figure 2 and Figure 3 were calculated in cases to compare with the corresponding siblings or friends for statistical analyses.

^aNomenclature from Taioli et al. (45); ^bHeterozygous and homozygous nonfunctional haplotypes. ^cHomozygous deleted gene.

Table 5. Conditional logistic regression analysis testing the association between metabolic variables and cases of TOS.

Variable	Cases/Siblings		Cases/Friends	
	Univariate OR (95% CI)	Multivariate OR (95% CI)	Univariate OR (95% CI)	Multivariate OR (95% CI)
Sex (female)	3.5 (1.4–8.7)	3.4 (1.3–8.6)	3.2 (1.5–7.2)	4.0 (1.7–9.8)
Age	1.1 (1.0–1.2)	1.1 (1.0–1.2)	0.9 (0.8–1.0)	0.9 (0.8–1.0)
<i>CYP1A1</i> (<i>C/D + C/M,A</i>) ^a	1.6 (0.6–4.0)		2.0 (0.8–5.0)	
<i>CYP2D6</i> (<i>wt/m + m/m</i>) ^b	1.6 (0.7–3.9)		1.3 (0.6–2.5)	
<i>NAT2</i> (<i>wt/m + m/m</i>) ^b	2.0 (0.4–10.9)	1.4 (0.2–8.7)	3.3 (0.9–12.1)	6.96 (1.4–33.2)
<i>GSTM1</i> (<i>GSTM1</i> -) ^c	1.2 (0.5–2.8)		0.7 (0.3–1.4)	
<i>GSTT1</i> (<i>GSTT1</i> -) ^c	1.2 (0.5–3.2)		0.8 (0.4–1.9)	
		*Log LR = 10.9 ($p = 0.012$)		*Log LR = 22.2 ($p < 0.001$)

In each matching group, univariate analysis yielded the corresponding OR for each independent variable. The mathematical model built with all the variables included yielded the final OR after multivariate analysis.

^aNomenclature from Taioli et al. (45); ^bHeterozygous and homozygous nonfunctional haplotypes. ^cHomozygous deleted gene. *Log of likelihood ratio tested as chi-square \times degrees of freedom ($n - 1$) variables included in the final model.

that the molecular structure of fatty acid anilides is similar to that of acetanilide and that fatty acid anilides exert a specific inhibition on benzo(a)pyrene 3-hydroxylation, a CYP1A1 marker (64). The fact that patients ingested both anilides and PAP esters adds more complexity to their biotransformation and/or mutual interaction.

Although a theory of free radicals that might involve glutathione conjugation was initially postulated (1,3), the present study clearly shows no involvement of glutathione transferase polymorphisms. Methemoglobinemia or tissue lesions such as those described by a typical aniline-acetanilide intoxication (3) have not been observed in TOS patients. Thus, if aniline had been released from the toxicant (i.e., by anilide/amide hydrolysis), it would have been circumvented by conjugation or other biotransformations. The potential cotoxicity of anilides is intriguing. For example, Berking et al. (65) recently reported a lethal wasting disease in A/J mice treated with oleylanilide. The disease observed in A/J mice, a slow acetylator strain, paralleled some of the human TOS disease features, whereas their homologous C57BL/6 strain (a fast acetylator) had no symptoms. The immunoresponse observed in these murine strains shows a profile similar to that described in TOS cases (33). The study of Berking et al. (65) suggests that acetylation may afford protection from reactive metabolites derived from oleylanilide, leading to toxicity. Using the same mouse strains, ongoing studies in our laboratory have shown that PAP oxidized metabolites at the aniline moiety (66) and oxidized acetanilide metabolites (67) were present in the animals' urine after intraperitoneal administration of ¹⁴C-labeled PAP or oleylanilide.

Genetic polymorphisms and epidemiological tools such as those used in this study might be useful in the examination of susceptibility factors in other diseases caused by toxicants. As far as TOS is concerned, the consideration of altered phase II metabolic pathways could be an important issue in obtaining an animal model that permits more in-depth analysis of the causal hypothesis. We believe that the present results strongly suggest the presence of a metabolic factor in the presentation of the disease. Further studies should be designed to confirm these findings. One of our greatest future interests is to clarify whether NAT2 mutations in TOS cases are associated with particular symptoms or sequelae, such as autoimmune disorders, because impaired acetylation has been associated with skin disorders produced after chemical exposure (68–71). Our laboratory is currently investigating metabolic and distribution pathways of TOS-implicated aniline derivatives in an animal model. We hope to

determine how other unexpected enzymes may contribute to TOS pathogenesis and how genetic polymorphisms may explain why people living in the same household had different degrees of morbidity.

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