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Assessment of CYP2C9, CYP2C19, and CYP2D6 Polymorphisms in Allergic Patients with Chemical Sensitivity

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Abstract
Background: Self-reported chemical sensitivity (SCS) is characterized by adverse effects due to exposure to low levels of chemical substances. The clinical manifestations of SCS are similar to the allergy, and a high percentage of individuals with both diseases have been found. Various genes, especially genes of importance to the metabolism of xenobiotic compounds, have been associated with SCS. ***Objectives:*** The purpose of this study was to investigate whether allergic individuals with chemical sensitivity differed from allergic patients without chemical sensitivity with regard to the distribution of genotype and phenotype of *CYP2C9*, *CYP2C19*, and *CYP2D6* polymorphisms. ***Methods:*** A total of 180 patients were enrolled for this study. A questionnaire was employed to collect information on individual chemical sensitivity, while the Skin prick test and the PATCH test were used to verify the presence of an allergic condition against inhalants or contact allergens, respectively. For the evaluation of the *CYP2C9*, *CYP2C19*, and *CYP2D6* polymorphisms, we used a strategy based on the amplification of the entire gene coupled to direct genomic DNA sequencing analysis. ***Results:*** Overall, a total of 15 different *CYP2C9*, *CYP2C19*, and *CYP2D6* haplotypes were identified in our population. If the 5 *CYP2C9* and the 2 *CYP2C19* identified alleles correspond to the previously described ones, 4 of the 8 *CYP2D6* haplotypes, detected in the study group, present new SNPs combinations. These new suballeles were categorized as *CYP2D6*2M* Salento Variant 1, *CYP2D6*35B* Salento Variant 2, *CYP2D6*41* Salento Variant 3, and *CYP2D6*4P* Salento Variant 4 due to the presence of the key SNPs 2,850 C>T, 31G>A, 2,988 G>A, and 1,846 G>A, respectively. When the allergic individuals are divided into 2 groups according to their SCS score, we

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observed that the distribution of the *CYP2D6* phenotypes was significantly different between the 2 groups. ***Conclusions:*** Our idea is that the application of the questionnaire that we have adopted has enabled us to diagnose a degree of chemical sensitivity, which results as comorbid of the allergic disease and in which a condition of poor or intermediate metabolizes for the detrimental *CYP2D6* alleles, could represent a discriminant between the chemical sensitivity and the health state.

Introduction

In the past few years, more attention has been paid to pathological conditions consisting of aberrant responses triggered by exposure to low doses of environmental pollutants or xenobiotics, in concentrations far below average reference levels admitted for environmental toxicants [1].

The most appropriate collective definition for these conditions seems to be "sensitivity-related illnesses." This state includes the hyper reactivity condition to diverse chemical excitants referred to as chemical sensitivity or just CS. The symptoms reported by CS subjects involve multiple organs. The reactions span from a mild (e.g., slight headache, sneezing, rash, dizziness) to a severe (e.g., incapacitating fatigue, pain, weakness, intestinal symptoms, heart palpitations, panic attacks) range. Some of the clinical manifestations of CS are similar to those related to the allergic condition, and a high percentage of individuals with both diseases have been described [2–5]. Moreover, the diagnosis of the allergic condition, in a patient, can be performed by in vivo tests (skin and provocation tests) and laboratory-based serologic analyses for IgE, since the etiology of allergy is known. Conversely, no diagnostic tools for CS are available, and the presence of the condition can only be established by a questionnaire and defined as self-reported chemical sensitivity (SCS).

Several modes of action have been suggested to explain CS, with the most commonly discussed theories involving the immune system, central nervous system, olfactory and respiratory systems, as well as an altered metabolic capacity.

In this context, several studies have been conducted to prove that CS might be due to the inherited impairment of xenobiotic metabolic capacity through phase I [6] and phase II enzymes [7] even thought this hypothesis is far from being demonstrated [8]. However, the most exhaustive study was conducted by Caccamo et al. [9] investigating on the distribution of selected Cytochrome P450 (CYP450) genetic variants in 2 cohorts of patients, coming from different Italian regions. The 2 groups of patients were diagnosed with Multiple Chemical Sensitivity (MCS) and Suspected MCS (SMCS) on the basis of a questionnaire score containing 10 common environmental exposures. One group of healthy Italian subjects is included in the analysis. Significantly higher frequency of CYP polymorphisms in patients compared to controls was found, supporting the idea that CYP genetic variants might represent a genetic risk factor for CS.

Cytochromes P450 (CYP450) are a superfamily of heme proteins that catalyze the oxidative transformation of a wide variety of structurally diverse compounds, including endogenously synthesized compounds such as steroids and fatty acids, as well as exogenous compounds such as drugs and environmental agents [10]. The human genome comprises 57 putatively functional CYP450 genes and 58 pseudogenes, which are classified according to sequence homology into 18 families and 44 subfamilies (http://drnelson.uthsc.edu/human.P450.table.html). Enzymes belonging to the CYP2-family have a role in the metabolism of the majority of drugs and other xenobiotics.

A very large number of genetic polymorphisms are confined in the coding region as well as in the regulatory regions of the *CYP2D6*, *CYP2C9*, and *CYP2C19* genes, leading to differences in the activities of these enzymes. To date, 50 distinct alleles have been reported for the *CYP2C9* gene and 30 for the *CYP2C19* gene, whereas *CYP2D6* shows the greatest impact of genetic polymorphisms among all major drug-metabolizing CYPs, with 105 distinct alleles currently reported. The Human Cytochrome P450 (CYP) Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/) has listed the allelic variants for the 3 cytochromes with their respective impacts on each enzyme's function defined as absent, decreased, or increased. The CYP2 genetic polymorphisms have an important clinical impact because they account for the large interindividual variability in drug clearance and responses to drug therapies in medical practice. For this reason, CYP2 genetic polymorphisms divide the population into 4 phenotypes: poor metabolizers (PM), intermediate metabolizers (IM), or ultrarapid metabolizers according to their ability to metabolize drug substrates with respect to the normal function defined as extensive metabolizers (EM) [11]. In addition, the PM condition confers a genetic predisposition to drug-induced adverse effects (ADR), which encompasses all of the adverse events related to the drug administration.

Even though the association between a given *CYP2C9* and *CYP2C19* genotype and its related phenotype is well

defined, phenotype prediction, according to the allele combination for the *CYP2D6* gene, is still ambiguous.

The sometimes observed lack of correspondence between the genotypes and phenotypes suggests the existence of additional genetic variants in the *CYP2D6* gene yet to be discovered. Recently, the search for regulatory *CYP2D6* polymorphisms has identified a specific SNP (rs5785550) located approximately 115 Kb downstream of the *CYP2D6* gene that increases the CYP2D6 transcription more than twofold [12, 13]. The inclusion of this variant in the *CYP2D6* genotype results in a more accurate phenotype prediction due to the robust effect of this SNP on the CYP2D6 mRNA/protein expression.

The present study was planned to determine the incidence of SCS in a group of allergic subjects from Southern Italy (Salento) in order to investigate the relationship between the two conditions. In addition, the distribution of the common *CYP2D6*, *CYP2C9*, and *CYP2C19* variants in SCS and allergy patients was also evaluated. Our study identified new allelic variants for the *CYP2D6* gene.

Materials and Methods

Study Design

A sample of 154 Italian consecutive hypersensitive patients received at the "Unità di Cura IMID Unit per le Malattie Infiammatorie Croniche Immuno-mediate e Ambiente-correlate, Presidio di Campi Salentina (Lecce)" over a period of 6 months (June–December 2012), was invited to participate in this study and examined by specialized clinicians. All the participants were informed about the experimental procedure and the purpose of the study and they signed a written consent. Subjects with a diagnostic history of cancer, chronic obstructive lung disease, cardiovascular disorder, or diabetes were excluded from the study. Additional exclusion criteria were the misuse of alcohol or misused exposition to any chemical (e.g., chemical workers, vehicle spray painter), which was assessed by asking the individuals. At the end, a total of 100 patients were enrolled for this first part of the study. In order to get a more meaningful statistic additional group of 80 subjects were selected later (September–December 2018) by the same medical staff at the POLISMAIL Unit (Specialistic Unit of Allergic and Immunological Diseases) in Lecce. In addition, 80 volunteer healthy individuals were recruited among the staff members of participant institution and chosen according to the criteria of absence of clinically diagnosed allergic disturbances or chemical sensitivity and no drug consumption since, at least, 2 months before the blood collection. At the end of the selection procedure, 63 individuals were enrolled as the control group (group C).

The questionnaire proposed by Schnakenberg et al. [7] was used to collect information on individual chemical sensitivity. This questionnaire is the simplified version of the EESI (Environmental Exposure and Sensitivity) questionnaire developed by Miller and Mitzel [14] and validated by Miller and Prihoda [15], which has been demonstrated to be an excellent tool, with a higher sensitivity and specificity for the screening of SCS patients. The rating scale used to define the severity of their sensitivity for each of the 10 chemicals for the original questionnaire was 0–10, whereas in the questionnaire that we adopted the rating scale was 1–3 (1 = no symptoms, 2 = moderate symptoms, 3 = disabling symptoms), avoiding the excessive use of subjective evaluations. Therefore, a minimum of 10 (all chemicals give no symptoms) and a maximum of 30 points (all chemicals give disabling symptoms) are achievable for each patient. However the severity of the symptoms for each chemical agent was evaluated from the medical staff with a second index that allows the patient to respond with greater awareness about their intensity at environmental exposures. The second index consists of 4 questions related to: (1) symptoms present for at least 6 months; (2) presence of at least 2 symptoms affecting 2 different organ; (3) symptoms causing lifestyle changes; and (4) symptoms are reduced when the chemical agent is removed. Only patients who respond positively to all 4 questions are classified with a rating scale of 3 for each chemical.

According to the achieved score, subjects with a score of >20 were defined as "sensitive" to common chemicals, individuals with a score of ≤20 were classified as "nonsensitive." The scores that we adopted are in accordance with the scores of EESI. Subjects achieving a score of 10–20 or 21–30 points using our questionnaire correspond to scores of 0–50 or 51–100 in the EESI questionnaire. The questionnaire was administered twice at an interval of 15 days and only those patients who provided consistent answers were considered for the study.

To verify the presence of an allergic condition against inhalants or contact allergens, the individuals were submitted to the skin prick test (SPT) and the serological analysis of specific immunoglobulin E as well as to the PATCH test.

The panel of SPT extracts comprised a variety of regionally perennial and seasonal aeroallergens. SPTs were performed by placing a drop of the test solution on the skin. SPT sites were wiped clean and a resulting wheal-and-flare response can be measured in 10–20 min. Histamine solution (10 mg/mL) was used as a positive control, and the diluent (HSA in 50% glycerol) was used as a negative control. A positive reaction was characterized as 3 mm or greater than that of the negative control. Serological quantification of specific IgE titers was conducted by radioallergosorbent tests (RAST). The PATCH tests, versus contact allergens of the European standard series, were applied to the shoulder of the patients. The reading was done 96 h after the application to avoid doubts about sensitization, defining as positive tests the ones with intensity marked by 2 or 3 crosses.

Individuals positive to the allergic tests were definite as allergic patients and classified according to the test response as "Prick/RAST positive," "Patch test positive," and "Prick/RAST and Patch test positive." Subjects who were negative to the allergic tests were defined as nonallergic patients.

Sample Collection

Blood samples were collected into tubes containing 0.1% EDTA. All personal identifiers were removed; thus, the samples were tested anonymously. The genomic DNA was extracted using the ArchivePure DNA Blood kit (5-PRIME, Hamburg, Germany), according to the manufacturer's recommended protocol. DNA was quantified spectrophotometrically at 260 nm, aliquoted, and stored at –20 °C until assayed. This study was approved by our Institutional Ethics Committee. All participants were informed

about the experimental procedure and the purpose of the study, and a written consent was obtained from each subject.

Genotype Evaluation

For the evaluation of the *CYP2C9*, *CYP2C19*, and *CYP2D6* polymorphisms, we used a strategy based on the amplification of the entire gene coupled to direct genomic DNA sequencing analysis.

Eight pairs of primers were designed to amplify the 9 exons with flanking intronic regions of both the *CYP2C9* and *CYP2C19* genes (online suppl. Table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000/497322) with classical PCR conditions. To avoid coamplification of pseudogenes, 3 pairs of primers (U1bis/L2, U6/L6 and U7/L8) chosen in a region of non-homology were designed to amplify the entire *CYP2D6* gene from position +1 to +4,575 (online suppl. Table S2). Because of the large fragments, initial amplification product was used as a template for nested PCR amplifications. To detect the genetic variants placed in the exon 1, in the intron 1, and in the exon 2, two nested-PCR (U1ter/L66 and U2bis/L2bis) protocols were developed starting from the U1bis/L2 PCR. For the exons 3–4 and 5–6 variants, the nested PCR U3bis/Bst and U4bis/L6bis, respectively, were generated from the U6/L6 PCR. The *CYP2D6* exons 7, 8, and 9 were amplified with nested PCR using primers U7s/L7, starting from the U7/L8 amplicon. The primers were designed on the reference sequences NG_008385 (*CYP2C9* gene), NG_008384 (*CYP2C19* gene), and AY545216 (*CYP2D6* gene).

PCR amplifications were carried out in a total reaction volume of 25 μL, with each reaction containing 50 ng of genomic DNA, 10 pmol of each primer, 320 μM dNTPs, 0.5 U 5PRIME DNA polymerase (5-PRIME, Hamburg, Germany), and 1× Reaction Buffer. The reaction cycle conditions consisted of an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at varying temperatures (online suppl. Table S1 and S2), and 30 s extension at 68 °C, with a final extension at 68 °C for 5 min.

The nested PCR conditions were identical to those used during original PCR amplification, except that the cycle number was changed to 25 cycles. About 1.0 μL of the original PCR product was used as template. After amplification, the generated 21 PCR products were purified by SureClean (Bioline) and used directly for both strands DNA sequencing by a commercial service. DNA sequence data were processed and analyzed using the blast program (http://www.ncbi.nlm.nih.gov/BLAST) with respect to the reference sequences. The generated sequence data also served to ensure that the reaction conditions used did not amplify any of the associated isoforms or pseudogenes.

Assessment of New CYP2D6 Subalelles

To verify the reliability of the new *CYP2D6* suballeles, the *CYP2D6* gene was amplified from position –1,343 to position +4,575 using the primer pair U66 (5′-TGAAGGTTGTAGTGAGCCGAG-3′)/L8, from patients homozygous for the new *CYP2D6*4* and *CYP2D6*35* suballeles, respectively, and from *CYP2D6*2M*/new*41 suballele heterozygote individual. The PCR was performed in 25 L containing 100 ng of DNA, 1×Pfu-DNA polymerase reaction buffer (Agilent Technologies, La Jolla, CA, USA), 0.2 mM of each dNTP, 0.4 mM of forward and reverse primers, and 2.5 units of Pfu-Turbo Hotstart DNA polymerase (Agilent Technologies). Amplification consisted of an initial denaturation step at 95 °C for 5 min followed by 35 amplification cycles (95 °C for 30 s, annealing at 55 °C for 30 s, and 72 °C for 2 min) and a final incubation at 72 °C for 10 min. A 30-deoxyadenosine overhang was added to blunt-ended amplicons of the homozygotes individuals by incubation with 1.0 unit of Platinum Taq DNA Polymerase (Invitrogen) at 72 °C for 10 min. These products were purified and cloned into the StrataClone TA-vector as per the manufacturer's instructions. For each sample, 6–10 colonies were propagated and bidirectionally sequenced using M13 and T7 vector-specific primers. All plasmid sequence data were analyzed and compared to the reference sequence to detect the presence of variants. The amplicon of the heterozygote subject was instead directly sequenced.

ARMS-PCR

In order to recognize the rs5758550 A/G polymorphism, we employed a tetra-primer amplification refractory mutation system (tetra-primer ARMS-PCR) [16]. The method consists in an allele-specific (AS) PCR based on the amplification of both alleles in the same test tube. Two AS inner primers, one for each allele of the polymorphism, and 2 outer primers were designed the web-based program accessible from http://probes.pw.usda.gov/batchprimer3/[17]. To enhance the specificity of the reaction, in addition to the first mismatch at the 3′ end of AS primers, an extra mismatch is also deliberately introduced at the third position from the 3′ end of each of the 2 inner AS primers.

All the allele-specific segments differed sufficiently in size to be distinguished by agarose gel electrophoresis. Primer sequences, annealing temperature, are shown in online supplementary Table S3.

In our hands, better PCR conditions were a 25-μL final volume of 10 mM Tris, pH 8.4, 50 mM KCl, 1.75 mM $MgCl_2$, 0.2 mM of each dNTP, 50 ng of genomic DNA, 25 pmol inner primers and outer primers, and 1U Taq DNA polymerase (5-PRIME, Hamburg, Germany). The amplification was performed for 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at the appropriate temperature for 30 s, and extension at 68 °C for 30 s. An initial denaturation step at 95 °C for 5 min and a final extension step at 68 °C for 5 min were also performed. PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide. To ascertained sensitivity and specificity, some results obtained with tetra-primer ARMS-PCR were compared with the sequence obtained with the 2 external primers.

Allele Categorization and Phenotype Prediction

We classified the alleles into clinically distinct categories for each of the 3 genes examined as loss-of-function, decrease function, or fully function, based on the defined molecular properties of the variant genes and reported in the CYP website (http://www.imm.ki.se/CYPalleles/). The combination of any 2 alleles represents the patient's diplotype. The diplotypes were then collapsed into 3 predicted phenotype categories EM, IM, or PM, according to the Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines for the *CYP2C9* [18] and the *CYP2C19* genes [19].

Briefly the phenotype classification system for the *CYP2C9* gene is as follows: patients with 2 functional alleles (*CYP2C9*1*) are categorized as EM. Individuals carrying one or 2 of the decreased functional alleles (*CYP2C9*2*, *CYP2C9*11*, *CYP2C9*12*, or *CYP2C9*18*) are considered IM and PM, respectively. For the *CYP2C19* gene, the phenotype classification system is as follows: the presence of 2 functional alleles (*CYP2C19*1*) confers the EM

phenotype, 2 of the loss-of-function alleles (*CYP2C19*2*) confer the PM phenotype, and the presence of only one copy of the loss-of-function allele confers the IM phenotype.

The *CYP2D6* metabolizer phenotype was inferred from genotype information based on the activity score system recommended by the CPIC guidelines for the *CYP2D6* gene [20]. Functional alleles with activity levels comparable to the *CYP2D6*1* reference allele were given a value of 1, while reduced function and nonfunctional alleles received values of 0.5 and 0, respectively. The CYP2D6 activity score was used to assign the phenotype as follows: patients with a diplotype corresponding to an activity score of 0 are classified as PM, those with a score of 0.5 are IMs, and those with a score from 1.0 to 2.0 are EM.

Statistical Analysis

Continuous data are expressed as mean ± SD; categorical data are expressed as percentage. Differences between the mean of the continuous data were evaluated by Student *t* test. Differences in allele and genotype frequencies and other categorical data between cases and controls were compared with Fisher's exact test. *p* values of ≤0.05 were considered significant.

Results

Detection of CYP2C9, CYP2C19, and CYP2D6 Polymorphisms and Their Frequency in Our Cohort

Given the variation of *CYP2D6*, *CYP2C9*, and *CYP2C19* allele distribution in populations of different ethnic and geographic origins [21, 22], we first carried out a systematic polymorphism analysis of the 3 genes in our cohort.

To recognize the *CYP2C9* and *CYP2C19* polymorphisms occurring in the 100 recruited patients from the 154 initially interviewed, their genomic DNAs were amplified with intron-specific primers across all 9 exons, followed by direct sequencing (see Materials and Methods). The resulting sequences were manually analyzed by comparison with the reference sequences, and the designations of all *CYP2C9* and *CYP2C19* alleles refer to those defined by the Cytochrome P450 Allele Nomenclature Committee (http://www.cypalleles.ki.se). Five different haplotypes for the *CYP2C9* gene were found. Beyond the wild-type *CYP2C9*1* haplotype, we found the *CYP2C9*2* (430T), the *CYP2C9*11* (1003T), the *CYP2C9*12* (1465T), and the *CYP2C9*18* (1075C/1190C/1425T) haplotypes, all defined as decreased function alleles.

In addition to the wild type, surprisingly the only other allele that we identified for the *CYP2C19* gene was the common loss-of-functional *CYP2C19*2* (681A) haplotype.

To identify the CYP2D6 genetic variations, the genomic DNA of the patients was amplified from the position +1 to +4,575 of the gene by using 3 pairs of primers. The 3 amplicons were subjected to a nested-PCR to generate 6 smaller fragments coupled with direct sequencing (see Materials and Methods). Eight different haplotypes were found in the 200 scrutinized alleles. The first is the wild-type haplotype without any differences with respect to the reference sequence. We will refer to this one as the *CYP2D6*1* allele. We also found a variant of the *CYP2D6* gene containing the key polymorphism 2,850T, which we identified from the human CYP Allele website as the *CYP2D6*2M* haplotype with a normal enzymatic activity. Other haplotypes present in our group are the loss-of-function *CYP2D6*3* (2549Adel) and the decreased function *CYP2D6*9* (2615–18AAGdel) and *CYP2D6*64* (100C>T; 1,023C>T) haplotypes. It was not possible to assign any of the haplotypes from 3 other samples, due to the presence of new SNPs combinations not reported on the website. One of those corresponds to the *CYP2D6*2M* allele definition but includes the 2,988G>A variation. The other one matches to the *CYP2D6*2M* haplotype but includes the 31G>A variant. The 31G>A and the 2,988G>A variants are "key" SNPs of the normal *CYP2D6×35* and the decreased function *CYP2D6*41* haplotypes, respectively. The last one was a sequence that could correspond to the *CYP2D6*4P* haplotype (1,846G>A), although with the absence of the 2,576C>T and the 3,435C>A substitutions.

To verify the reliability of these new haplotypes, we amplified a promoter/exon9 amplicon, encompassing from position –1,343 to +4,575 of the *CYP2D6* gene by long-PCR, which we cloned and sequenced from a patient homozygous for the new *CYP2D6*4P* and *CYP2D6*35* alleles.

Because in our court we have not found homozygous subjects for the new *CYP2D6*41* allele, we used genomic DNA from a *CYP2D6*2M*/new *CYP2D6*41* allele heterozygous individual for the amplification of the gene by the long PCR. The obtained amplicon was then sequenced directly. The sequence analysis of the cloned fragments confirms the presence of new suballeles in our population that could be identified as hybrids between *CYP2D6*2M* and *CYP2D6*35B* or *CYP2D6*2M* and *CYP2D6*41*. We considered these haplotypes as variants of the *CYP2D6*35B* and *CYP2D6*41* due to the presence of the key SNP 31G>A and 2,988G>A, respectively. Therefore, we appoint them as *CYP2D6*35B* Salento Variant 2 (*CYP2D6*35B* SV2) or *CYP2D6*41* Salento Variant 3 (*CYP2D6*41* SV3), respectively. Moreover, we found 2 modifications in the 5′-UTR of the *CYP2D6*2M* haplotype present in our group compared to the *CYP2D6*2M* of the Human CYP Allele website, consisting of an insertion of 5A at the –1237_–1,236 position and the absence of the –750_749delGA. We have named this new suballele as *CYP2D6*2M* Salento Variant 1 (*CYP2D6*2M* SV1). The se-

Table 1. Structure of the newly discovered CYP2D6 alleles in our cohort

Position	CYP2D6 *2M SV1	CYP2D6 35B *SV2	CYP2D6 *41 SV3	CYP2D6 *4P SV4	Region
–1,237_–1236 insA	insAAAAA	insAAAAA	insAAAAA	insAAAAA	
–1,235 A>G	G	G	G	G	
–1,094 delA				delA	
–1,002 A>G	A	A	A	G	5'UTR
–1,000 G>A	A	A	A	A	
–740 C>T	T	T	T	C	
–678 G>A	A	A	A	G	
–244 A>G	A	A	A	G	
31 G>A	G	A	G	G	Exon 1
100 C>T	C	C	C	T	
CYP2D7P gene conversion in Intron 1	Conversion	Conversion	Conversion	–	
310 G>T	T	T	T	T	Intron 1
746 C>G	G	G	G	G	
843 T>G	G	G	G	G	
974 C>A	C	C	C	A	
984 A>G	A	A	A	G	Exon 2
997 C>G	C	C	C	G	
1,661 G>C	C	C	C	C	Exon 3
1,846 G>A	G	G	G	A	Intron 3
2,097 A>G	A	A	A	G	Intron 4
2,850 C>T	T	T	T	C	Exon 6
2,988 G>A	G	G	A	G	Intron 6
3,384 A>C	C	C	C	C	
3,582 A>G	A	A	A	G	Intron 7
3,584 G>A	A	A	A	G	
3,790 C>T	T	T	T	C	
4,180 G>C	C	C	C	C	Exon 9
4,401 C>T	C	C	C	T	3'UTR
4,481 G>A	A	A	A	G	

quence analysis also confirmed the presence of a new CYP2D6*4P suballele in our population with a difference in the 5′-UTR as well as in the coding region compared to the *CYP2D6*4P* allele present on the Human CYP Allele website. We identified this new allele as *CYP2D6*4P* Salento Variant 4 (*CYP2D6*4P* SV4). The detailed structure of the newly discovered *CYP2D6* suballeles in our population is reported in Table 1. The novel sequences were deposed onto the NCBI GenBank (Ac. No KU531563-66).

The allele frequencies of the haplotypes for all of the genes, detected from the 100 individuals, were reported in Table 2. The *CYP2C9*1*, *CYP2C9*2*, *CYP2C9*18*, and *CYP2C19*2* alleles are the only present in our cohort for the *CYP2C9* and *CYP2C19* genes.

As expected, the wild-type alleles *CYP2D6*1* and *CYP2D6*2*, represented solely by the *CYP2D6*2M SV1* in our group, were the most common alleles found in our subjects. *CYP2D6*4P SV4* and *CYP2D6*41 SV3* (the only suballele found in our population for these haplotypes) are, instead, the only consistently detrimental alleles found in our groups. We *also* found very low frequencies for the other haplotypes *CYP2D6*3*, *CYP2D6*9*, *CYP2D6*35B* SV2 (the only suballele found in our population for this haplotype), and *CYP2D6*64*.

Table 2. *CYP2C9*, *CYP2C19*, and *CYP2D6* allele frequencies detected in our cohort

Gene	Allele	Frequencies, %
CYP2C9	*1	85.5
	*2	7.0
	*18	6.5
	*12	0.5
	*11	0.5
CYP2C19	*1	80.0
	*2	20.0
CYP2D6	*1	41.0
	*2	24.0
	*3	2.0
	*4	17.0
	*41	10.5
	*9	0.5
	*35	3.5
	*64	1.5

CYP2C9, CYP2C19, and CYP2D6 Phenotype Classification

For the *CYP2C9* and *CYP2C19* gene, we determined the metabolizer status in all of the patients as deduced from the genotype according to the CPIC guidelines for the *CYP2C9* [18] and the *CYP2C19* genes [19] and described in Materials and Methods section.

In the 100 patients of the study population, the CYP2C9 EM phenotype was found in 75 patients, while 4 individuals who were either homozygotes for the decreased functional *CYP2C9*2* and *CYP2C9*18* alleles or heterozygotes for *CYP2C9*2/*18* alleles were classified as PM. The remaining 21 subjects, heterozygotes for the decreased-function alleles exhibit the IM phenotype As could be expected by the exclusive presence of *CYP2C19*2* in the studied population, the main genotype is the wild-type *CYP2C19*1/*1* (62%). Conversely, unique genotypes related to IM and PM phenotypes, observed here, were *CYP2C19*1/*2* (36%) and *CYP2C19*2/*2* (2%), respectively (online suppl. Table S4).

For the classification of the CYP2D6 metabolizer phenotype inferred from the genotype, we applied the activity score (AS) system (see Methods). The effects of the most common *CYP2D6* alleles have been already described [8, 23, 24]. The assignment of the CYP2D6 activity score for each allele was based on the value proposed by the Human Cytochrome P450 Allele Nomenclature Committee [20] and on recent studies evaluating the level of CYP2D6 mRNA expression [12, 13].

It has been found that the 2,850C>T (rs16947) substitution in exon 6, currently considered to have no effect, is instead associated with a reduced enzymatic activity, due to the destruction of exonic splicing enhancer, resulting in exon skipping [12]. As a consequence, the reduced enzymatic activity established for the *CYP2D6*41* allele containing both the 2,850T and the 2,988G variations is due to the 2,850T rather than the 2,988G. The transcriptional activity of the alleles bearing the 2,850T variant can be returned to normal levels only in the presence of a change in in the downstream enhancer SNP rs5785550 [12, 13].

Due to the robust effects of rs5785550 on the CYP2D6 expression, we included the rs5785550 identification in the genotyping panel of our patients for a better prediction of CYP2D6 enzymatic activity.

To this purpose, we performed an ARMS-PCR with 2 AS internal primers, one for each allele of the polymorphism, and 2 external primers. To ascertain sensitivity and specificity, some results obtained with ARMS-PCR were compared to those coming from direct sequencing of the amplicon obtained by using external primers. We obtained a 100% concordance between the results of the 2 methods. In Table 3, we reported the rs5785550 A>G variation in relation to the haplotypes identified in our study population. In our cohort, all of the *CYP2D6*1* alleles, as well as the *CYP2D6*4P* SV4, *CYP2D6*3*, *CYP2D6*9*, and *CYP2D6*64* haplotypes, have the rs578550A variation. Among the haplotypes carrying the 2,850T variation, all of the *CYP2D6*35B* SV2 alleles have the rs578550G polymorphism, indicating a CYP2D6 mRNA level compared with the reference. On the contrary, the *CYP2D6*41* SV3 alleles have the rs578550A variation with a decreased mRNA level. Among the 32 *CYP2D6*2M* SV1 alleles, 4 (12.5%) have the rs578550A variations. We have given a value of 0.5 to these alleles indicating them as *CYP2D6*2M* SV1A. The remaining 28 *CYP2D6*2M* SV1 haplotype with the rs578550G variation were classified as *CYP2D6*2M* SV1G allele as they were given with a value of 1.

The combination of alleles with the related activity score was next used to assess the diplotype and the phenotype classification of our patients (online suppl. Table S5). In our selected study population, 4 participants were classified as PMs, 7 as IMs, 89 were predicted to be EMs. Of the 89 genotype combinations with the EM status, 6 exceeded a 5% frequency in the population, accounting for a 70% frequency overall. The most common allele combinations are *CYP2D6*1/*1* and *CYP2D6*1/*2M* SV1G, with an activity score of 2. We also found diplotypes that carry the *CYP2D6*41* SV3 or *CYP2D6*4P* SV4 alleles in combination with either the *CYP2D6*1* or

Table 3. Summary of the reported *CYP2D6* alleles and their effect on CYP2D6 protein

Haplotype	Major nucleotide variation	dbSNP number	Effect on CYP2D6 protein	Activity score[#]
*1	–	–	–	1
*2M SV1G	rs5785550A>G 2,850C>T 4,180G>C	rs5785550 rs16947 rs1135840	Increase expression R296C (decrease expression) S486T	1
*2M SV1A	2,850C>T 4,180G>C	rs16947 rs1135840	R296C (decrease expression) S486T	0.5
*3	2,549delA	rs35742686	Frameshift	0
*4P SV4	100C>T 1,846G>A 4,180G>C	rs1065852 rs3892097 rs1135840	P34S splicing defect S486T	0
*9	2615_2617delAAG	rs749023275	K281del	0
*35B SV2	rs5785550A>G 31 G>A 2,850C>T 4,180G>C	rs5785550 rs762378491 rs16947 rs1135840	Increase expression V11M R296C (decrease expression) S486T	1
*64	100C>T 1,023C>T 4,180G>C	rs1065852 rs28371706 rs1135840	P34S T107I S486T	0.5
*41 SV3	2,850C>T 2,988G>A 4,180G>C	rs16947 rs28371725 rs1135840	R296C (decrease expression) Splicing defect S486T	0.5

[#] For the classification CYP2D6 variants see Methods.

*CYP2D6*2M* SV1G haplotype, with an activity score of 1.5 and 1, respectively. The IM status is determined only by one combination, while the PM condition is stated by the presence of 2 different null alleles.

Distribution of the CYP2C9, CYP2C19, and CYP2D6 Gene Polymorphisms in Allergic Patients with and without SCS

The 180 individuals of the study group were subjected to the SPT and to the serological analysis of specific immunoglobulin Es as well as to the PATCH test in order to check for the presence of an allergic condition against inhalants or contact allergens, respectively. One-hundred and ten of the 180 patients were positive for the allergic test, 45 were negative, whereas 25 decided not to undergo tests and were eliminated from subsequent analysis. The 110 allergic patients were asked to complete the questionnaires to define their chemical sensitivity. Based on the median self-reported chemical-related sensitivity score (≤20 and >20), the 110 individuals were categorized into 2 groups. The individuals with a high sensitivity score (>20) were defined as sensitive (SCS, 34 patients, group A), and individuals with a score ≤20 were classified as nonsensitive (non SCS, 49 subjects, group B). Twenty-seven patients provided contradictory answers to the questionnaire and for this reason they were excluded from the successive analysis. The 45 nonallergic patients were also asked to complete the questionnaire providing 10 individual exhibiting SCS and 24 no SCS. Eleven patients gave contradictory responses. Therefore, in our population, the 77% of SCS patients (34/44) are allergic, whereas about 31% of allergic patients are also SCS (34/110).

After the above-reported evaluation, we performed the analysis of the *CYP2C9*, *CYP2C19*, and *CYP2D6* genes by using the strategy already describe, for genotype the additional patients and the 63 healthy individuals as well.

The main features of the subjects from the A, B, and C groups are shown in Table 4. We observed a slight differ-

Table 4. Demographic data, clinical characteristics, and haplotype frequencies[#] in the patients distributed in our groups

	Group A (n = 34)	Group B (n = 49)	Group C (n = 63)
Gender, *n* (%)			
Male	1 (2.9)	5 (10.2)	6 (9.5)
Female	33 (97.1)	44 (89.8)	57 (90.5)
Average age, years			
Means ± SD	50.9±12.4	48.8±14.1	42.5±7.3.
Median (range)	49 (30–82)	48 (20–86)	42 (30–54)
Allergy, n (%)			
Prick-Rast test	4 (11.8)	8 (16.3)	–
Patch test	20 (58.9)	29 (59.2)	–
Prick-Rast/patch test	10 (29.3)	12 (24.5)	–
Chemical score			
Means ± SD	22.6±1.4	12.7±1.5	10.0
Haplotype, n (%)	(n = 68)	(n = 98)	(n = 126)
CYP2C9*2	5 (7.3)	8 (8.2)	3 (2.4)
CYP2C9*18	6 (8.8)	6 (6.1)	12 (9.5)
CYP2C19*2	11 (16.1)	21 (21.4)	6 (4.8)
CYP2D6*4P SV4	15 (22.0)	15 (15.3)	12 (9.5)
CYP2D6*41 SV3	12 (17.6)	8 (8.2)	9 (7.1)

[#] Raw p values of the statistical analysis are reported in the text.

ence, no statistical significance, in the distribution of the gender between the 2 categories of SCS scores (p = 0.20). Age was equally distributed between the 2 groups with no significant differences compared to the median age (p = 0.73). The subjects exhibited allergy against inhalants (Prick-RAST test) or contact allergens (Patch test) or both (Prick-RAST/Patch test) with a similar frequency in both groups (p = 0.41, p = 0.33 and p = 040, respectively). The chemical sensitivity score was instead significantly different between the 2 groups (p = 0.0003). While no difference can be observed in the distribution of sex in the control group with respect the other 2 groups (p = 0.22 C vs. A; p = 0.57 C vs. B), the average age is lower and significantly different (p = 0.001 C vs. A, p = 0.03 C vs. B). No allergic condition or chemical sensitivity was reported for the subjects of C group.

When we investigated the frequency of the 5 more frequent (>5%) detrimental *CYP2C9*, *CYP2C19*, and *CYP2D6* alleles found in our study population, we observed an almost similar frequency for the *CYP2C9*2* (p = 0.50 A vs. B) and *CYP2C9*18* (p = 0.35 A vs. B) alleles in the 2 groups. The *CYP2C19*2* was more frequent in B group than in A group, but this difference was not significant (p = 0.26 A vs. B). The *CYP2D6*4P* allele was more frequent in A group compared to the B group, but the difference was not significant (p = 0.18 A vs. B) as well. The *CYP2D6*41* allele shows, instead a higher frequency with a p value very close to being significant in the A group with respect to the B group (p = 0.05 A vs. B). In the control group, the frequencies of the single alleles were lower in comparison to the A and B groups with differences that reach the significance for the *CYP2C19*2* (p = 0.009 C vs. A and p = 0.0001 C vs. B) and *CYP2D6*4P* (p = 0.01 C vs. A and p = 0.09 C vs. B) alleles. The difference for the *CYP2D6*41* allele was significant in the control in comparison with the A group (p = 0.02 C vs. A), but was not significant in comparison with the B group (p = 0.48 C vs. B). No significant was the differences for the *CYP2C9*18* (p = 0.54 C vs. A and p = 0.24 C vs. B) allele, whereas the difference for the *CYP2C9*2* allele was not significant with respect to the A group (p = 0.54 C vs. A) and significant if compared to the B group (p = 0.047 C vs. B).

In addition, we identified all of the diplotypes and the phenotypes for *CYP2D6*, *CYP2C9*, and *CYP2C19* genes in the 2 categories of SCS scores and in the con-

Table 5. Distribution of the CYP2D6, CYP2C9, and CYP2C19 diplotypes and phenotypes prediction in our groups

Diplotype	Activity score	Phenotype[#]	Frequency		
			A (n = 34)	B (n = 49)	C (n = 63)
CYP2D6 *1/*1	2	EM	7	8	22
CYP2D6 *1/*2G	2	EM	5	6	10
CYP2D6 *1/*35	2	EM	–	1	–
CYP2D6 *2G/*2G	2	EM	1	8	4
CYP2D6 *2G/*35	2	EM	–	–	2
CYP2D6 *35/*35	2	EM	1	–	–
CYP2D6 *2A/*2G	1.5	EM	–	1	–
CYP2D6 *2G/*64	1.5	EM	–	1	–
CYP2D6 *1/*41	1.5	EM	5	5	2
CYP2D6 *2G/*41	1.5	EM	2	3	7
CYP2D6 *2A/*41	1	EM	1	–	–
CYP2D6 *2G/*4	1	EM	2	3	6
CYP2D6 *35/*4	1	EM	1	1	–
CYP2D6 *1/*4	1	EM	1	9	5
CYP2D6 *2G/*3	1	EM	–	1	–
CYP2D6 *1/*3	1	EM	–	–	3
CYP2D6 *1/*9	1	EM	–	–	2
Total		EM	26 (76.4)	47 (96.0)	63 (100)
CYP2D6 *4/*41	0.5	IM	4 (11.8)	–	–
CYP2D6 *4/*64	0.5	IM	–	1 (2.0)	–
CYP2D6 *4/*4	0	PM	3	1	–
CYP2D6 *4/*3	0	PM	1	–	–
Total		PM	4 (11.8)	1 (2.0)	–
CYP2C9 *1/*1		EM	23 (67.7)	37 (75.5)	47 (74.6)
CYP2C9 *1/*2		IM	5	5	4
CYP2C9 *1/*18		IM	4	5	12
CYP2C9 *1/*12		IM	1	–	–
Total		IM	10 (29.4)	10 (20.4)	16 (25.4)
CYP2C9 *2/*2		PM	–	1	–
CYP2C9 *2/*18		PM	–	1	–
CYP2C9 *18/*18		PM	1	–	–
Total		PM	1 (2.9)	2 (4.1)	–
CYP2C19 *1/*1		EM	24 (70.6)	29 (59.2)	56 (88.9)
CYP2C19 *1/*2		IM	9 (26.5)	19 (38.8)	7 (11.1)
CYP2C19 *2/*2		PM	1 (2.9)	1 (2.0)	–

Values are n (%).
[#] For the phenotype classification see Materials and Methods.
EM, extensive metabolizers; IM, intermediate metabolizers; PM, poor metabolizers.

trol group (Table 5). Even though the frequency of the EM, IM, and PM CYP2C9 phenotypes is similar in the 3 groups, we observed an increment of the IM CYP2C19 phenotype in the group B with respect to SCS patients and control group. Only one individual exhibited an IM phenotype for the *CYP2D6* gene in the B group (2.0%), whereas we found 4 IM individuals in SCS (11.8%). The PM phenotypes appear in only 1 patient of the B group

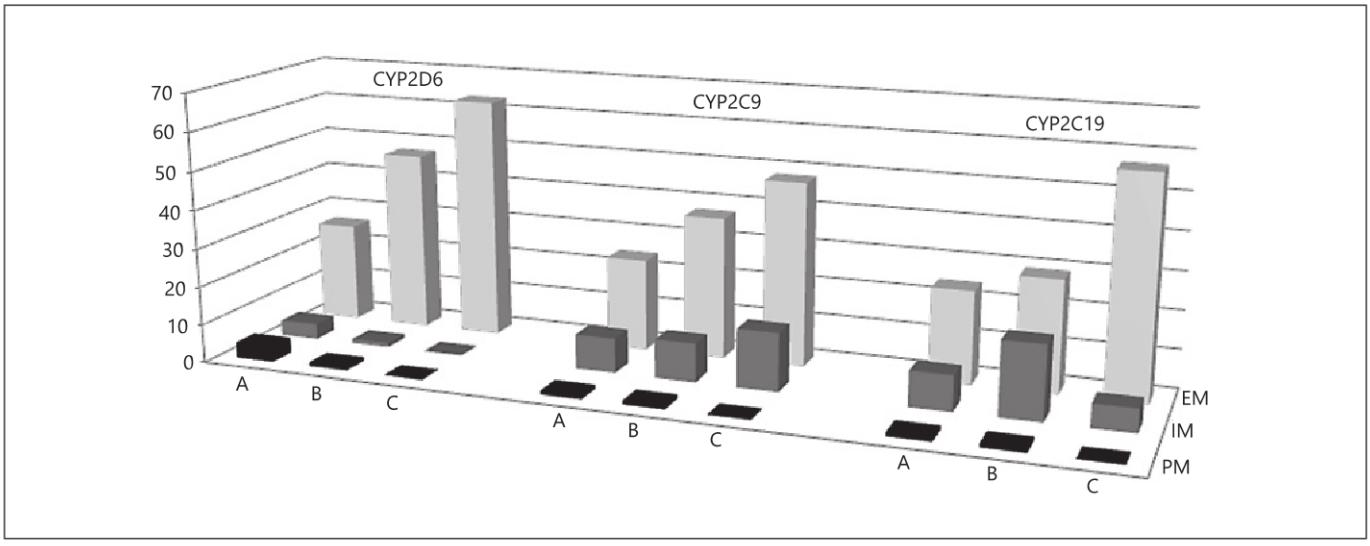

Fig. 1. Graphical representation of the distribution of EM, IM, and PM phenotypes for *CYP2D6*, *CYP2C9,* and *CYP2C19* genes in A (SCS), B (non-SCS) and C (control) groups. Raw *p* values of the statistical analysis are reported in the text. EM, extensive metabolizers; IM, intermediate metabolizers; PM, poor metabolizers.

(2.0%) while emerge in 4 individual (11.8%) in the SCS group. No IM and PM phenotypes were found in the group C.

As a consequence, the difference in the distribution of the 3 CYP2C9 phenotypes between the 3 groups was not significant ($p = 0.8$ A vs. B; $p = 0.49$ A vs. C; $p = 0.28$ B vs. C), whereas the difference in the distribution of the CYP2C19 phenotype was significant in the A and B group compared to C group ($p = 0.02$ A vs. C; $p = 0.0001$ B vs. C). Interestingly, the difference in the distribution of the 3 CYP2D6 phenotypes was significant if the SCS group was compared to non-SCS group and to control ($p = 0.02$ A vs. B; $p = 0.0001$ A vs. C), but was not significant when the B group was compared to group C ($p = 0.18$ B vs. C). In Figure 1, we show a graphical representation of the phenotype distributions of the 3 genes in the 3 groups.

On the basis of these results, we evaluated the clinical manifestations of subjects exhibiting the IM and PM phenotypes for *CYP2D6* gene related to the chemical sensitivity condition. The IM and PM individuals have a chemical score ranging from 21 to 24 with disabling symptoms for 5 exposures to various solvents and cleaners, indoor air contaminants, and agricultural chemicals with a greater severity of symptoms as muscular weakness and fatigue, respiratory distress, persistent headache, arthralgia, abdominal plain, loss of concentration, and mood disorders.

Discussion

Chemical sensitivity and allergy are often defined as closely related diseases in which adverse reactions to a normally innocuous environmental antigen were established [2]. Clinical manifestations are similar in the 2 conditions, and a high percentage of individuals with both diseases have been found. Comorbid self-reported allergic disease was significantly higher in MCS patients (84.0%) than those of the general population (30%) in Japan [3]. More recently, Katerndahl et al. [4] compared a subset of clinical patients, who met criteria for chemical sensitivity, with the nonchemical sensitivity patients, revealing a significant difference for the self-reported allergies (53 vs. 40%, respectively). The prevalence of chemical sensitivity between allergic and nonallergic groups was also different (19.7 and 11.3%, respectively) in a Korean population [5]. Also in our study, the prevalence of chemical sensitivity among allergic patients was found to be relevant: 77% of the SCS patients are also allergic. Therefore, a relationship between allergy and chemical sensitivity can be assumed. Convinced of the need to find genetic markers for a better diagnosis of SCS, we evaluated the distribution of *CYP2C9, CYP2C19*, and *CYP2D6* variants in our patients.

The most consistent result that we found was a difference, at limit of significance, in the frequency of the *CYP2D6*41* allele in SCS group compared to non-SCS

group, assessing allergic individual patients to the A or B group on the basis of a score obtained for the SCS questionnaire. In particular, a genetic background positive for the presence of the *CYP2D6*41* haplotype (*2,850T/2,988A*) with rs5785550A was more frequent in SCS patients with respect to non-SCS ones (Table 4). However, the distribution of EM, IM, and PM phenotypes for the CYP2D6 gene was significantly different, comparing the SCS group to non-SCS group, with the IM individuals, exhibiting the *CYP2D6*41/*4* diplotype, not recorded in non-SCS group. On the contrary, the frequency of the variants and the phenotypes for the *CYP2C9* and *CYP2C19* genes were not statistical different between the 2 groups (Table 5; Fig. 1). A higher frequency of the *CYP2C19*2, CYP2D6*4P*, and *CYP2D6*41* variants in A group compared to control was found. Instead, the *CYP2C9*2* and *CYP2C19*2* alleles exhibit a higher frequencies significantly different in the B group with respect to the control. Thus, our results support the hypothesis that gene variants for *CYP* genes might be related to SCS.

CYP2D6, *CYP2C9*, and *CYP2C19* genes have been investigated in relation to chemical sensitivity in other population. In Caccamo's study [9], a modified QEESI score of 10 common environmental exposures and 10 major symptoms allowed them to identify patients with MCS (20≤ score ≤30), SMCS (suspected MCS) (10≤ score ≤20), or healthy individuals (0≤ score ≤10) and in which was found a higher frequency of *CYP2C9*2*, *CYP2C9*3*, *CYP2C19*2*, *CYP2D6*4*, and *CYP2D6*41* alleles in the patients compared with controls. CYP2D6*4 was the most represented gene variants in all subgroups of SCS, while the *CYP2D6*41* allele was absent in the control population. Genetic background positive for the presence of one or more *CYP* variants was adopted from the same group as marked, together with questionnaire, to selected SCS patients for successive investigation [25]. The finding of a role of the CYP variants in the SCS condition was not confirmed by Berg et al. [8], but in this study, few *CYP2D6* variants were analyzed and the *CYP2D6*41* allele was not determined. In addition, the chemical score of SCS was not well defined.

Another previously reported study on a Canadian population [6] suggests that individual with higher *CYP2D6* activity (EM phenotype) have an increased risk for MCS compared to individuals carrying 2 nonfunctional alleles of the *CYP2D6* gene (PM phenotype). The discordance with our data may be prevalent due to different employed technical approaches, since the study was done using the less sensitive PCR-RFLP technique. In addition, the University of Toronto Health Survey (UTHS) is the self-administered questionnaire, used to assess the SCS condition based on 171 symptoms, 85 exposures linked to symptoms, and 9 specific "features" described in 6 previously published MCS case definitions. Overall, the comparison of the outcomes between the different studies is difficult to apply due to: (i) the difference in the method used for the *CYP* genotyping; (ii) the ethnogeographic characteristics of an examined population; and (iii) the use of different questionnaires.

Our idea is that various levels of chemical sensitivity may exist and that the application of the questionnaire that we have adopted has enabled us to diagnose certain chemical sensitivity that results as comorbid of the allergic disease and in which a condition of poor and IMs for the detrimental *CYP2D6* alleles, in particular, *CYP2D6*41*, could represent a discriminant between the "intolerance syndrome" and the health state. The availability of a small number of patients requires a degree of caution in the interpretation of data and supports the explorative nature of our study. However, it should be underlined that the patients were carefully selected, and the genotyping techniques, that we used, have 100% of sensitivity and specificity. The haplotypes are well defined, it includes the promoter region, and phenotypes are deducted from the genotypes according to the more recent definition [18, 19, 20]. All these settings could represent a new direction of investigation.

The allergic response is initiated by the uptake of allergens by the antigen-presenting cells to naive T cells, thereby directing them in favor of a Th2 phenotype. The active Th2 cells coordinately upregulate the expression of a cluster of proinflammatory cytokines that are involved in the class-switching of B cells to IgE synthesis, the recruitment of mast cells, and the maturation of eosinophils and basophils, which are the main mediator-secreting effector cells of the allergic response [26]. On the other hand, recent studies have increasingly implicated various inflammatory stimuli, first of all proinflammatory cytokines, as causing a reduction in the activities and expression levels of CYPs [27, 28].

Our hypothesis is that in patients with a compromised *CYP2D6* gene, the presence of elevated levels of proinflammatory cytokines due to the allergic condition may further reduce the levels of activity of the cytochrome, predisposing patients to chemical sensitivity. This situation could particularly affect the extrahepatic tissues, especially those that are the portals of entry for foreign

compounds, such as the respiratory and the gastrointestinal tracts or the nasal mucosa, which also express xenobiotic-metabolizing CYPs [29].

Furthermore, because it was reported that there are differences in allelic distributions in populations of different ethnic group and geographic areas [30, 31], we first determined the *CYP2C9*, *CYP2C19,* and *CYP2D6* genotype profile of our 100 individuals from Salento, a Southern Italy region. A total of 12 different variant alleles were identified in the study population.

Thus far, the 4 identified *CYP2C9* variants have been well characterized. The frequencies of the *CYP2C9*2* and **3* alleles and diplotypes derived from them have slightly lower frequencies with respect to those found in other European populations [18], but they represent the 2 most common variants with decreased enzyme function also in our population.

In our cohort, we identified the most common loss-of-function *CYP2C19*2* allele with a frequency slightly higher than that of the European populations [19]. If the *CYP2C9* and *CYP2C19* identified alleles correspond to the previously described ones, some difference can be observed in our population in relation to the *CYP2D6* suballeles context.

In our population, we found the 3 well-characterized *CYP2D6*3, CYP2D6*9,* and *CYP2D6*64* variants and 4 new subvariants that we appointed *CYP2D6*2M* SV1, *CYP2D6*35B* SV2, *CYP2D6*41* SV3, and *CYP2D6*4P* SV4 due to the presence of key SNPs 2,850C>T, 2,850C>T/31G>A, 2,850C>T/2,988G>A, and 1,846G>A, respectively. These suballeles are the only representatives of the *CYP2D6*2*, *CYP2D6*35*, *CYP2D6*41,* and *CYP2D6*4* haplotypes.

Interesting is the structure of the *CYP2D6*35B* SV2 and *CYP2D6*41* SV3 alleles, which are characterized by the presence of the SNPs 31G>A and 2,988G>A, respectively, but otherwise identical to *CYP2D6*2M* SV1. A similar *CYP2D6*2M/*41* hybrid haplotype was also found in the Sardinian population [30].

The *CYP2D6*2*, **35* and **41* haplotypes are characterized by the key SNP 2,850C>T, for which the current system for translating the CYP2D6 genotype into phenotype is not optimally calibrated. Initially identified as a variation without any effect on the enzyme activity, the 2,850T allele has recently been associated with reduced activity if not supported by the change in the downstream enhancer SNP rs5785550A>G [13, 14]. For this reason, we have incorporated the typing of the enhancer SNP in the CYP2D6 genotyping. In our cohort, while the *CYP2D6*2M* SV1 allele is often (87.5%) linked to the rs5785550G variant, the *CYP2D6*41* SV3 allele is always (100%) linked to the rs5785550A variant, predicting an allele with a reduced enzymatic activity. On the contrary, the *CYP2D6*35B* SV2 allele, which always carries the rs5785550G variant, was considered to convey normal enzyme activity.

Both the detrimental *CYP2D6*4* and **41* haplotypes were largely represented in our population with a frequency comparable to that of African populations rather than Caucasian, although the *CYP2D6*41* has not consistently been determined by its key SNP 2,988G>A across studies. This means that the *CYP2D6*2* and *CYP2D6*41* alleles cannot be discriminated, and this may lead to over estimation of the *CYP2D6*41* allele [20]. A higher *CYP2D6*41* frequency (17.8%) was found in Sardinia [30]. These results indicate a higher prevalence of this allelic variant in the Mediterranean area compared to Northern Europe.

*CYP2D6*41*, in addition to *CYP2D6*4*, can be considered as an important mutant allele in our group, contributing to the incidence of the IM phenotype, while *CYP2D6*4* alone accounts for the PM phenotype. The incidence of IM and PM in our population is very low (5 and 4%, respectively) as estimated for other Caucasian populations [31]. On the basis of these observations, we propose the genotyping of the 2,988G>A variant for the *CYP2D6* gene as diagnostic biomarker for discriminating the SCS condition. Because of the absolute association 2988A/rs5785550A, the 2,988G>A polymorphism can serve as a tagging SNP for identifying haplotypes containing 2,850T and rs57855550A, thus predicting reduced CYP2D6 mRNA/protein activity [13].

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Statement of Ethics

All study participants or their guardians provided informed consent. The study design was approved by the appropriate Ethics Review Board.

Disclosure Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

M.P.B., M.M., and S.M.: designed research and wrote the paper. S.D.A.: provided all the genomic study. F.M.: provided technical assistance in cloning experiments. M.M., S.M., D.M., and I.V.: received the patients, collected and analyzed all the clinical data. All authors have read and approved the final manuscript.

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