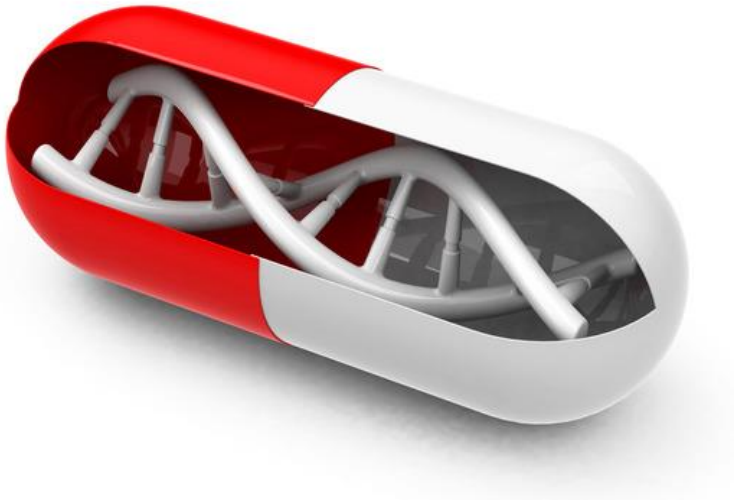

Updating the study of pharmacogenetic polymorphisms in the Portuguese Roma

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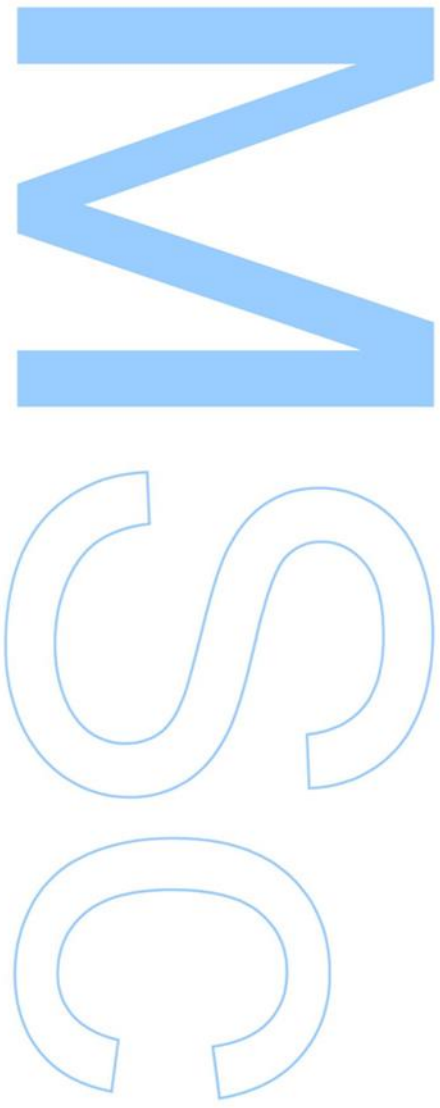

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Resumo

Polimorfismos em genes que codificam enzimas metabolizadoras de fármacos (EMFs) são os maiores influenciadores da variabilidade interindividual no que diz respeito à resposta a fármacos. Dentro das EMFs, o citocromo P450, família 2, subfamília D, polipéptido 6, CYP2D6, é uma das mais importantes pois é responsável pela metabolização de 25 a 30% de todos os fármacos prescritos, incluindo antidepressivos, antipsicóticos, antiarrítmicos, bloqueadores β, anti-tumorais, entre outros. A atividade enzimática do CYP2D6 varia bastante entre indivíduos, principalmente devido a variações genéticas no gene codificante, resultando em consequências clínicas significativas para o metabolismo de fármacos e também riscos individualizados no que toca a efeitos adversos ou alterações na resposta pretendida. Apesar de várias populações já terem sido estudadas relativamente ao CYP2D6, ainda existem algumas que foram escassamente analisadas, como o caso dos ciganos europeus.

Os ciganos são um caso de estudo populacional interessante que ainda está pouco estudado em vários aspetos. Durante o seu período migratório, era normal ocorrer a fragmentação em grupos mais pequenos, mantendo na mesma as suas práticas endógamas. Do ponto de vista genético, isto leva a elevados efeitos de deriva genética, diversidade genética reduzida, elevado linkage disequilibrium e fluxo genético limitado entre grupos.

Assim, e atendendo à escassez de estudos farmacogenéticos nos ciganos, neste estudo pretendemos caracterizar a população de ciganos portugueses, através do estudo de *Single Nucleotide Polymorphisms* (SNPs) com interesse farmacogenético, e para avaliar se estes apresentam diferenças no que toca à resposta a fármacos, comparativamente à restante população portuguesa. Nesse sentido, 56 amostras de ciganos portugueses foram analisadas por reação de SNaPshot™, desenhado e otimizado para detetar 11 SNPs dentro do gene CYP2D6, que estão associados a alterações na resposta a fármacos. Para comparação de resultados, foram usados dados anteriormente publicados para a restante população portuguesa, bem como dados de outras populações de todo o mundo, para inserir os nossos resultados num contexto mundial.

Das variações estudadas, apenas uma não se encontrava de acordo com o equilíbrio de Hardy – Weinberg, mesmo após a correção de Bonferroni, que foi a posição

4180 G>C. Comparativamente à restante população portuguesa, os ciganos apresentam uma frequência mais elevada do CYP2D6*4, um dos alelos que apresenta atividade nula. Relativamente aos perfis metabólicos teoricamente definidos, foram encontradas diferenças entre os ciganos e os restantes portugueses, principalmente nos perfis *Poor Metabolizers* (PM), *Intermediate Metabolizers* (IM) e *Extensive Metabolizers* (EM).

Contudo, apesar de ter sido aplicada uma técnica de considerável resolução, em termos de SNPs, estudos futuros são necessários para aprofundar os resultados obtidos, preferencialmente com análise conjunta bioquímica da atividade enzimática. Para além de caracterizar melhor a população cigana portuguesa, será também importante ter dados de outras populações ciganas europeias, para que o conhecimento sobre os ciganos europeus permita extrair mais inferências relevantes do ponto de vista da farmacogenética.

Com o desenvolvimento deste estudo, contribuímos para um aprofundamento da caraterização da população cigana, ajudando a promover o conhecimento do ponto de vista farmacogenético, relativo ao CYP2D6, bem como a realçar a importância da farmacogenética no cenário clínico.

Palavras – Chave: Farmacogenética, Enzimas Metabolizadora de Fármacos, CYP2D6, SNP, População Cigana

Abstract

Polymorphisms in genes coding for drug-metabolizing enzymes (DME) are major players in interindividual variability in drug response. Among DME, cytochrome P450 family 2, subfamily D, polypeptide 6, CYP2D6, is one of the most important, because it is involved in metabolism of 25% to 30% of all prescribed drugs, including antidepressants, antipsychotics, anti-arrhythmics, β-blockers, cancer chemotherapeutics, among others. The enzymatic activity of CYP2D6 varies widely among individuals, mainly due to functional genetic variations at the encoding gene, resulting in significant clinical consequences for drug metabolism and individual risk of adverse events or drug efficacy. Although many populations have been studied regarding the CYP2D6, there are still populations scarcely studied, such as the Roma from Europe.

The Roma represent an interesting population case study that is still understudied in many aspects. During the Gypsy diaspora, it was normal the occurrence of group fragmentation into smaller communities, but still maintaining endogamous practices. From a genetic point of view, this leads to high genetic drift effects, reduced genetic diversity, high linkage disequilibrium and limited gene flow between these groups.

Therefore, due to these traits and to the scarcity of Pharmacogenetic studies in this population, in this present study, we intended to characterize the Portuguese Roma for Single Nucleotide Polymorphisms (SNPs), relevant from the Pharmacogenetics point of view, to evaluate if the Roma show any peculiarity regarding drug response, in comparison with the host population. In that sense, 56 samples from Portuguese Roma were analysed by SNaPshot™ reaction, designed and optimized to detect 11 SNPs located within the CYP2D6 gene, known to influence drug response. For comparative analysis, data from previous studies in the Portuguese host population was used, as well from several populations around the world, to put the data obtained in a worldwide context.

For the variations studied, only one SNP revealed significant deviation from the Hardy – Weinberg expectation, even after the Bonferroni correction, which was the 4180 G>C. Comparatively to the Portuguese host population, the Roma showed some differences, especially an increased frequency of the CYP2D6*4, an allele implying null enzymatic activity. Regarding the theoretical metabolic profiles, differences were found, especially the IM and PM profiles.

Even though we studied a set of SNPs considered to be of high resolution, further studies are needed to deepen the results here obtained, preferably with the joint analysis of biochemical assessment of enzymatic activity. Also, new studies are needed, not only in the Portuguese host population, but also in other European populations, with a larger number of SNPs, so that the knowledge regarding these populations and further comparative analysis can allow to extract more inferences relevant from the perspective of Pharmacogenetics.

With the development of the present study, we have contributed to deepening the characterization of Roma populations, helping to further the knowledge from the pharmacogenetic point of view regarding the CYP2D6, as well as to enhance the importance of Pharmacogenetics in the clinical setting.

Keywords: Pharmacogenetics, Drug-Metabolizing Enzymes, CYP2D6, SNP, Roma Population

Table of Contents

Acknowledgments ……..3

Resumo ……....4

Abstract ……....6

Table of Contents ……...8

Table Index ……10

Figure Index ……..11

Abbreviations ……12

I. INTRODUCTION …… 15

1. Pharmacogenetics ……15

1.1. Pharmacogenetic Applications ……16

1.2. Pharmacogenetic Challenges ……...17

2. Drug Metabolism ……18

2.1. Phase I of Drug Metabolism ……20

2.1.1. Cytochrome P450 system …… 20

2.1.1.1 Cytochrome P450 2D6 – CYP2D6 …….. 22

2.1.1.2 Worldwide Genetic Distribution ……25

3. Portuguese Roma ……..26

II. AIMS ……. 27

III. MATERIALS AND METHODS ……..27

1. Samples and DNA extraction …….. 27

2. PCR Design …… 28

2.1. Electrophoresis ……...30

3. SNP Genotyping ……30

4. Sanger Sequencing ……..…32

5. Data Analysis ……..33

IV. RESULTS AND DISCUSSION ……33

1. CYP2D6 Genotyping……..33

2. Inference of CYP2D6 diplotypes …… 36

3. Metabolic Profiles ……...39

4. Comparison with other populations……...42

V. FINAL REMARKS ...……...47

VI. BIBLIOGRAPHY ……...49

Table Index

Pages

Table 1 - Drugs metabolized by the CYP2D6 gene. 22

Table 2 - Primers used in the PCR amplifications. 29

Table 3 - Primers used in the SNaPshot™ reactions. 31

Table 4 - Primers used in the Sanger Sequencing reactions. 32

Table 5 – Results from the Hardy – Weinberg equilibrium test obtained for the 11 positions. 36

Table 6 – Results of Linkage Disequilibrium (L.D.) obtained for the 11 positions. 37

Table 7 - Haplotypes defining different CYP2D6 alleles, corresponding effects in enzyme activity and frequencies in the Portuguese Roma. 37

Table 8 - Frequencies of CYP2D6 genotypes in the Portuguese Roma population. 40

Table 9 – Frequencies of CYP2D6 variant alleles in different populations. 43

Figure Index

Page

Figure 1 - Schematic representation of drug metabolism in the human body. 19

Figure 2 - Representation of the different phenotypes in a population. 21

Figure 3 - Schematic representation of the CYP2D6 gene cluster. 23

Figure 4 – Schematic representation of different CYP2D6 gene arrangements 29

Figure 5 - Representation of the CYP2D6 exons and the different positions of the SNPs analysed 31

Figure 6 – Example of an Agarose Electrophoresis performed for the analysis of the whole gene and cases of gene deletion 34

Figure 7 – Example of an electropherogram performed in this study and respective predicted genotype 34

Figure 8 – Example of an agarose electrophoresis performed to determine the presence or not of duplication events 35

Figure 9 - Example of an agarose electrophoresis performed to determine the presence of the whole duplicated gene 35

Figure 10 - SNaPshot™ reaction performed to confirm the results of the whole duplicated gene approach 36

Figure 11 - Frequencies of theoretical metabolic profiles in the Portuguese Roma population 41

Figure 12 - Frequencies of theoretical metabolic profiles 47

Abbreviations

µL	Microlitre
µM	Micromolar
5th	fifth
10th	tenth
15th	fifteenth
AS	Activity Score
A	Adenine
β	Beta
B.C.	Before Christ
BLAT	Blast – like Alignment Tool
C – O	Carbon – Oxygen
C	Cytosine
ºC	Degree Celsius
CEPH	Centre Étude Polymorphism Humain
CNV	Copy Number Variation
CPIC	Clinical Pharmacogenetics Implementation Consortium
CYP/CYP450	Cytochrome P450
CYP1A2	Cytochrome P450 1A2
CYP2A6	Cytochrome P450 2A6
CYP2B6	Cytochrome P450 2B6
CYP2D6	Cytochrome P450 2D6
CYP2D7	Cytochrome P450 2D7
CYP2D8P	Cytochrome P450 2D8 Pseudogene
CYP2C19	Cytochrome P450 2C19

CYP3A4	Cytochrome P450 3A4
del.	Deletion
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DMEs	Drug Metabolizing Enzymes
EM	Extensive Metaboliser
et al.	et alli
Exo – SAP	Exonuclease I and Shrimp Alkaline Phosphatase
G	Guanine
GSH	Glutathione
H	Hydrogen
HGDP	Human Genome Database Panel
i.e.	in example
IM	Intermediate Metaboliser
In/Dels	Insertions/Deletions
kb	Kilobase
L.D.	Linkage Disequilibrium
min.	minute
mRNA	messenger Ribonucleic Acid
n	Sample Size
OH	Hydroxide
OligoCalc	Oligonucleotide Properties Calculator
PCR	Polymerase Chain Reaction
PM	Poor Metaboliser
REP	Repetitive Segment
®	Registered

Ref	Reference
SBE	Single Base Extension primers
s. d.	standard deviation
sec.	second
SNP	Single Nucleotide Polymorphism
T	Thymine
™	Trademark
UCSC	University of California, Santa Cruz
UM	Ultrarapid Metaboliser
v.	version
WGS	Whole – Genome Sequencing
XMEs	Xenobiotic Metabolizing Enzymes

I. INTRODUCTION

1. Pharmacogenetics

The term pharmacogenetics is often used to describe a relatively new field of genetics that covers the study of how different gene variants affect drug response or, by other words, the study of the effect of heredity in human's drug response. Nowadays, pharmacogenetics is largely contributing to the emergence of personalised medicine, also called individualized or precision medicine that aims, in its narrow sense, to customize health care, with decisions and treatments tailored to each patient (1).

Primordial observations in this field go back to 510 B.C., when Pythagoras described that some individuals, after ingesting fava beans, showed dangerous reactions, when the rest of them did not (2).

In the 1950s, the introduction of therapeutic drug monitoring, generally defined as the clinical practice of measuring specific parameters at regular intervals that, with appropriate interpretation, directly influence drug prescribing procedures (3), represented the first systematic approach to individualize the treatment of patients that still has significant value in assessing drug therapy, particularly in psychiatry and severe infections (4, 5). With the unravelling of the structure of DNA and subsequent development of molecular biology, a new era was open to study the genetic bases of phenotypes associated with variability in drug response. Since then, it became increasingly well documented that genetic variations was an important determinant of interindividual variability in drug response (6).

The grand majority of genetic variations influencing drug response belong to the class of Single Nucleotide Polymorphisms (SNPs), which are variations in single nucleotides of the DNA sequence characterized by the occurrence in at least 1% of the population. SNPs are the most common polymorphisms in the human genome, accounting to more than 90% of the human genetic variations. Although most SNPs do not present effect on gene function, some have profound impact on the function of associated genes, whether the SNPs occur in the coding regions or at a significant distance from the transcription starting site of the gene (6). When such kind of variations are encountered in genes that encode enzymes responsible for absorption, distribution, metabolism or excretion of drugs, enzyme activity can be disturbed, leading to undesirable response to drugs which can be potentially fatal. Therefore, it is important to

study these polymorphisms, not only on a pharmacological perspective (which can, in a near future, lead to individualized treatments), but also on a forensic viewpoint, where it can be applied, together with Toxicology, to solve cases where the cause of death is inconclusive (7, 8).

1.1. Pharmacogenetics applications

With the development of health care and the rise of its costs, there has never been a greater need to reduce waste. In this context, ineffective treatments and adverse drug effects must urgently be minimized. Nowadays, the path to maximizing benefit and reduce negative effects seems to reside in stratifying patient populations into subgroups taking into account the patient individuality in response to a certain treatment since it is well known that an individual's response to a drug is affected by personal factors such as age, gender and genetics. Before a drug can be used in the clinical practice it must be subjected to clinical trials conducted to collect data demonstrating its safety and efficacy. Typically, however, clinical trials only give information regarding the "average patient", not taking into account that every individual has different backgrounds which can lead to a wide variability in drug response. It is now widely considered that pharmacogenetics must have a critical role in the clinical trials, specifically in the early stages of the treatment, by assisting to define the number of patients to analyse, to predict the efficiency of the therapy and to identify subgroups in which a distinct treatment is needed. Besides the contribution to the success of clinical trials, pharmacogenetics is also crucial in the clinical setting, where the use of genetic tests can give a prediction of drug response regarding a certain person before the treatment is started, allowing thus saving in expensive therapeutics that would probably not work and at the same time preventing levels of toxicity that sometimes cannot be measured by traditional methods. This happens, for instance, in oncological treatments, where the use of genetic tests can lead to the identification of various mutations in driver oncogenes that reduce the binding capacity of the drug and consequently low therapeutic success and/or resistance (5, 9-11).

Although pharmacogenetics has been preponderantly associated with the advantages it can provide in terms of clinical applications, it is also gaining ground in forensic sciences. Forensic pharmacogenetics is a relatively new and growing area of research. It is being increasingly applied in *post-mortem* cases, as an adjunct in the determination of the cause of death, namely through the analysis of certain genetic

variants in specific genes that encode proteins involved in drug metabolism. Pharmacogenetic approaches might help to infer if high levels of a specific drug detected in a corpse can be the cause of death due to overdose or due to inefficient metabolism caused by the presence of a certain genetic variation. As a matter of fact, acute drug intoxication is a major cause of unnatural deaths in occidental countries, usually requiring a forensic investigation to determine the reasons of the intoxication and consequently the likely explanation for the death. Among the most common drugs found in intoxication cases are psychiatric drugs and analgesics such for instance tramadol, an opioid analgesic commonly prescribed in the treatment of postoperative, dental, cancer, neuropathic and acute musculoskeletal pain. Since the enzyme CYP2D6 plays a predominant role in tramadol metabolism, and given that null variants in the encoding gene associated with enzyme inactivation can cause acute intoxication in patients administrated with inaccurate doses of the drug, CYP2D6 is being one of the most widely studied genes in forensic pharmacogenetics (7, 12).

1.2. Pharmacogenetics Challenges

Despite being well recognised the importance of pharmacogenetics, which continues to grow, particularly in the clinical practice and in the pipeline of drug development, pharmacogenetics also faces some setbacks. For instance, the evaluation of the clinical cost – effectiveness of a genetic test is critical. Even though there are studies evidencing the advantages of pharmacogenetic testing, in order to obtain valid, accurate and relevant cost-effectiveness estimates, reliable economic studies are required and this proves to be a challenge because often there is no hard clinical evidence regarding the effects of the test on the clinical utility (4). Furthermore, the information is not available for every genetic variant of pharmacogenetic relevance. Differences in costs can be substantial between countries, or even laboratories, and thus it is important to take this into account in scenario analysis, as well as the sensitivity and specificity of pharmacogenetics tests, which also represent another challenge since they can vary in function of the different ethnicities studied or genetic variations analysed (4).

To perform economic evaluations of pharmacogenetic tests is necessary to rely in relevant clinical data, which are often difficult to obtain. Until recently, evidence has been mainly provided by means of conventional clinical trials, but there is now a movement toward using observational studies instead of randomized trials. The Clinical

Pharmacogenetics Implementation Consortium (CPIC), based in the United States, promotes reviewed guidelines on how to use pharmacogenetics tests results to improve treatments based on evidence according to patients' medical records (13). Though the development of CPIC guidelines was a good step toward the implementation of pharmacogenetics tests in routine clinical practice, some fear that the assessment of pharmacogenetics might be obstructed because these guidelines "bypass traditional data requirements needed to support the use of a new intervention and move straight to producing recommendations for its use in clinical practice" (13). In other words, some people claim that the production of guidelines without information about, for instance, its effectiveness in a certain population, such as the one we can obtain through randomized trials, may still give insufficient information regarding cost effectiveness, not allowing policy makers to make reimbursement decisions easier. Therefore, there is the need to boost the development of a strong evidence - base for pharmacogenetics by alternative means that can satisfy the requirements of health care payers (4, 9, 13).

2. Drug Metabolism

Xenobiotics are foreign chemical substances which are not normally produced or expected to be within an organism. When these substances enter the human body, they pass through several biochemical transformations in order to be transformed in other metabolites, less harmful, minimizing thus aggression to the organism. The process by which these biochemical transformations occur is commonly known as xenobiotic or drug metabolism. One of the most important end results of the biochemical reactions that transform the foreign elements in products easier to be eliminated, is to avoid accumulation in the organism, thus preventing possible toxic reactions. Although drug metabolism typically inactivates drugs, they can also convert an inactive drug (called prodrug) into an active one, an active drug into an active or toxic metabolite, or an unexcretable drug into an excretable form (14, 15).

To process these foreign substances, many biochemical transformations occur that require the machinery of several enzymes, known as drug metabolizing enzymes or xenobiotic enzymes (DMEs or XMEs), responsible by different steps in the metabolic pathways. While DMEs can be synthetized in many organs, the most active one is the liver, where the grand majority of these enzymes are produced. Accordingly, it is also in the liver that many drugs begin to undergo extensive biotransformation. Its effectiveness

in the liver might be very high, so that only a small portion of the drug reaches the blood stream. This process - known as first pass effect or presystemic metabolism – whereby the concentration of a drug is greatly reduced before it reaches the systemic circulation, greatly influences the bioavailability of a drug (16, 17).

Drug metabolism is divided in three phases: phase I, in which DMEs introduce reactive or polar groups into xenobiotics, mainly by oxidation, hydroxylation and hydrolysis (Figure 1, PHASE 1); phase II, where these modified compounds are then conjugated into polar compounds, such as glutathione (GSH) and glucuronic acid (Figure 1, PHASE 2); and phase III, when the xenobiotic conjugates are further metabolized and excreted (Figure 1, PHASE 3). Although these phases can occur separately, they normally take place in succession. Whatever the process and phase of drug metabolism, the rate of biotransformation reactions is not the same for every individual, since differences in genetic profiles and environmental factors, such as age, gender, drinking and smoking habits, can account to wide intra-individual variability (17-19).

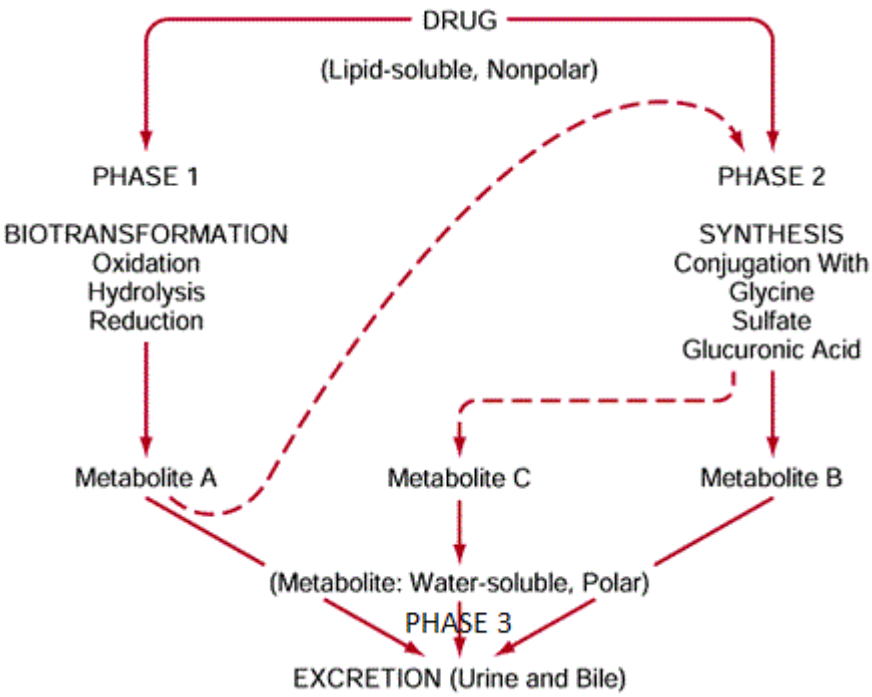

Figure 1 - Schematic representation of drug metabolism in the human body. The Drug Metabolism is divided in 3 Phases: Phase 1, where occurs the biotransformation of the xenobiotics; Phase 2, where occurs the synthesis of these xenobiotics; Phase 3, where occurs the excretion of the metabolites resultant from the biodegradation of the xenobiotics through the different pathways.

2.1. Phase I of Drug Metabolism

Drug Metabolizing Enzymes (DMEs) play central roles in the metabolism, elimination and/or detoxification of xenobiotics or exogenous compounds introduced into the body (17). In the phase I of Drug Metabolism, a variety of enzymes act to introduce reactive and polar groups into their substrates. Phase I reactions may occur by oxidation, reduction, hydrolysis, cyclization and decyclization, all occurring predominantly in the liver. For instance, through phase I oxidation, which involves the conversion of a C-H bond to a C-OH, a pharmacologically inactive product (prodrug) can be converted into a pharmacologically active compound, or also a nontoxic molecule can be transformed in a toxic one as illustrated by phase I metabolism of acetonitrile to glycolonitrile, which rapidly dissociates into formaldehyde and hydrogen cyanide, both of which are toxic (14, 17, 20). Most of the enzymes involved in phase I reactions belong to the large family of cytochrome P450 (CYP450) enzymes, including the CYP2D6.

2.1.1. Cytochrome P450 system

Cytochrome P450 (CYP450) is the generic name given to a large family of highly versatile enzymes involved in drug metabolism and response (15). Besides the preponderant role in xenobiotic metabolism, they also have important functions in other processes, including steroid and cholesterol biosynthesis, fatty acid metabolism and the maintenance of calcium homeostasis. CYP450 enzymes can be found abundantly in the endoplasmic reticulum of liver cells, but also in the gastrointestinal tract, lung and kidney, though showing lower levels of expression (17, 21).

There are 57 active CYP genes in the human genome, which are divided into 18 families, accordingly with their amino acid sequence similarities. The first three families (CYP 1-3) are mainly responsible by the metabolism of exogenous substances, such as drugs, whereas the CYP families with higher numbers are usually more involved in the metabolism of endogenous substances. CYP enzymes participate in 75-80% of all phase I – dependent metabolism and for 65-70% of the clearance of clinically used drugs (22). The CYP isoenzymes are named according to specific standards, which can be exemplified using the CYP2D6: CYP is the abbreviation for cytochrome P450, the

number “2” designates the family, the letter “D” stands for the subfamily and the number “6” represents a specific gene (23).

From all the active elements of the CYP family, CYP3A4 is responsible for about 50% of all CYP-dependent drug metabolism. In the encoding gene, more than 20 common genetic variants have been identified, most of them resulting in decreased enzymatic activity, which substantially contribute to the high variability among individuals regarding CYP3A4 – mediated drug metabolism, although other factors concur for such variability. On the other hand, nearly 40% of the metabolism of all clinical used drugs is carried out by CYP2C19 and CYP2D6 enzymes, whose genes are as well highly polymorphic. Other members, like CYP1A2, CYP2A6 and CYP2B6, that contribute greatly to xenobiotic metabolism, are also encoded by highly polymorphic genes (22).

Due to the polymorphic nature of the CYP enzymes, they are usually associated with the phenotypic variation elicited in drug-treated subjects, which is much dependent on levels of enzymatic activity. Accordingly, individuals are often described to be ultrarapid, extensive, intermediate and poor metabolisers (Figure 2). Ultrarapid metabolisers (UM), usually carry more than two copies of alleles conferring together greater than normal enzymatic activity; intermediate metabolisers (IM) are heterozygous for one defective and one functional allele or for 2 partially defective alleles; poor metabolisers (PM) carry two defective alleles and extensive metabolisers (EM) carry two alleles with normal function of the CYP enzyme. Therefore, depending on the phenotype of each individual, the drug metabolism can vary, leading to alterations in drug effectiveness (15, 22, 24).

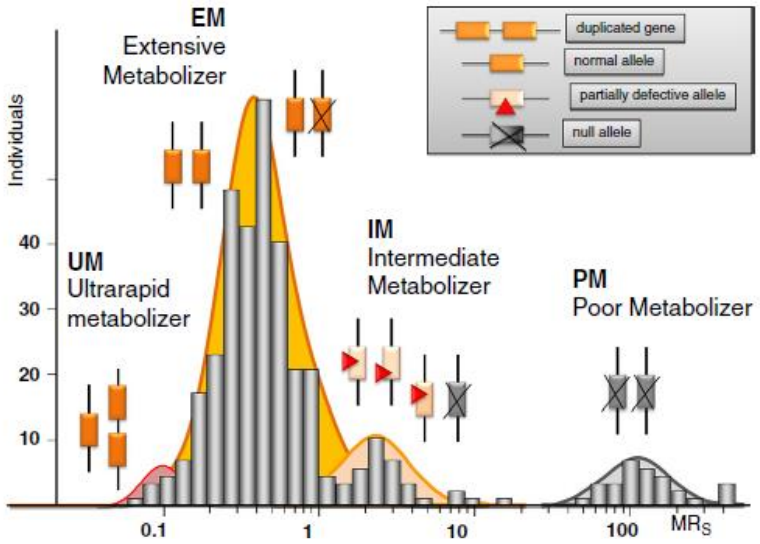

Figure 2 - Representation of the different phenotypes in a population. Ultrarapid Metabolizers present more than 2 copies of the gene, Extensive Metabolizers present 2 copies of the normal gene, Intermediate Metabolizers present 1 copy with normal function and 1 with reduced function r both with reduced function and Poor Metabolizers present no functional copies of the gene. Adapted from Krammer *et al.* (2009)

2.1.1.1. Cytochrome P450 2D6 – CYP2D6

So far, CYPD26 has been one of the most studied pharmacogenes, because it presents the greatest impact of genetic polymorphism among all major CYP enzymes, due to its wide spectrum of genetic variants (from null alleles to several-fold gene amplification), comparably little influence by environmental and non - genetic factors, and its extraordinarily broad substrate selectivity (15).

CYP2D6 contributes to the metabolism of up to 25% of all clinically used drugs from virtually all therapeutic classes (25). It is involved in the Phase I of the drug metabolism, working through the various reactions described in point 2.1. According to He *et al.* (26), CYP2D6 acts on more than 160 drugs, including tricyclic antidepressants, serotonin-selective reuptake inhibitors, opioids, and antiemetics, but also antiarrythmics, β-blockers, antihistamines, and antiviral agents. In Table 1 are listed the most common clinically used drugs metabolized by CYP2D6, as well as some known drugs that may function as inhibitors of CYP2D6 activity.

Table 1 – Drugs metabolized by the CYP2D6 gene. Adapted from Department of Medicine, Indiana University's website (27)

SUBSTRATES				**INHIBITORS**
Beta-Blockers	**Antidepressants**	**Antipsychotics**	**Others**	
Carvedidol	Amitriptyline	Haloperidol	Aripiprazole	Bupropion
S - metopropol	Clomipramine	Risperidone	Atomoxetine	Fluoxetine
Propafenone	Desipramine	Thioridazine	Codeine	Paroxetine
Timolol	Duloxetine		Dextromethorphan	Quinidine
	Fluoxetine		Doxepine	Duloxetine
	Imipramine		Flecainide	Amiodarone
	Paroxetine		Mexiletine	Cimetidine
			Ondansetron	Aripiprazole
			Oxycodone	Diphenhydramine
			Tamoxifen	Chlorpheniramine
			Tramadol	Clomipramine
			Venlafaxine	Doxepin
				Haloperidol
				Methadone
				Ritonavir
				Terbinafine

The CYP2D6 gene is located on chromosome 22q13.1, in a region containing the entire CYP2D gene cluster, which besides CYP2D6 also harbours 2 pseudogenes, CYP2D7 and CYP2D8P (Figure 3). The pseudogene CYP2D7 is expressed as mRNA in the liver, but due to an insertion in the first exon disrupting the reading frame, protein production is null. In the case of the CYP2D8P, it's a true pseudogene which has accumulated several gene-disrupting mutations (15). The expression of CYP2D6 is mostly confined to the liver, although low levels of protein have been shown in extrahepatic tissues like the gastrointestinal tract and in the human brain.

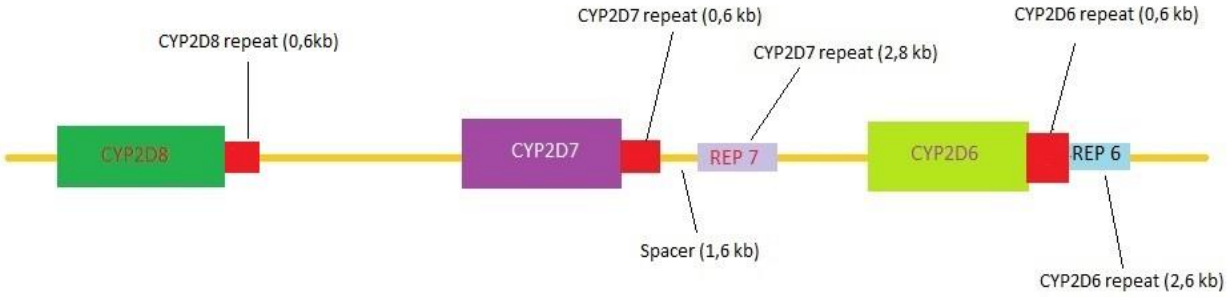

Figure 3 - Schematic representation of the CYP2D6 gene cluster. In dark green, purple and light green, we have the representation of the CYP2D8, CYP2D7 and CYP2D6, respectively. The CYP2D7 spacer is located between a common 0,6 kb repeat (marked as red) and the 2,8 kb REP 7 sequence, a repetitive segment specific of the CYP2D7 pseudogene (marked as grey). The 0,6 kb repeat is found downstream of all genes. The 2,8 kb REP 6 sequence, a repetitive segment specific of the CYP2D6 gene, is marked as a blue. Adapted from Krammer *et al.* (2009) and Gaedigk *et al.* (2007)

CYP2D6 gene contains 9 exons spanning ~4,4 kb, and encodes a protein with 497 amino acids, although other alternatively spliced transcripts resulting in different isoforms have been reported (http://grch37.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000100197;r=22:42522501-42526908). There are currently more than 100 distinct alleles and a grand number of rare variants described in the Human Cytochrome P450 (CYP) Allele Nomenclature Database - (http://www.cypalleles.ki.se/cyp2d6.htm). Many result in absent or non – functional protein (i.e. CYP2D6*3, *4, *5, *6), in decreased (i.e. CYP2D6*9, *10, *17, *41) or increased (i.e. CYP2D6*1XN, *2XN) expression. Other alleles present normal function (i.e. CYP2D6*1 or Wild type and CYP2D6*2) (15, 28-30).

The majority of these alleles result from sequence variations like SNPs and small In/Dels within the CYP2D6 gene. However, structural variants are also commonly

detected in the entire CYP2D locus, because the locus is very prone to unequal crossing over between the highly homologous regions it contains. Accordingly, complete or partial deletions and duplications were shown to occur often, meaning thus that copy number variation involving the CYP2D6 gene is quite common. In addition, other products of recombination within the CYP2D cluster account to the diversity at the locus. It follows that both sequence and structural variations may originate alleles conferring no, decreased, normal or increased function, which in the whole are highly responsible for the wide range of levels of enzymatic activity across individuals. One of the extremes contains the so-called poor metabolizers, individuals with two non-functional alleles who cannot metabolize or bioactivate drugs through CYP2D6, while the other extreme contains the ultra-rapid metabolizers, who are those carrying at least one increased function allele (i.e., two or more copies of a functional allele on one chromosome) in addition to a normal-function allele. The groups of poor and ultra-rapid metabolizers are at the highest risk for experiencing dose-related adverse events or treatment failure, depending on the drug administered (25).

Given the clinical relevance of CYP2D6, numerous genotyping strategies have been developed (28, 29) with the main goal of be able to predict the metaboliser phenotype of each tested individual. However, the design of genotyping assays for CYP2D6 is very challenging, once is necessary to deal with the major problem of detecting not only the multitude of alleles defined by sequence variation in a gene that is highly polymorphic, but also the structural variants that might be present in combination with any other CYP2D6 allele. For this reason, even with the most accurate genotyping methods, much caution is needed to make phenotypic predictions from genotypic data (29). Indeed, the reliability of phenotype prediction from genotype data is strongly influenced by the alleles interrogated in the typing assays. Unless using complete resequencing approaches, many alleles altering enzymatic activity might escape detection, resulting in frequencies of default alleles misleadingly overestimated (25).

This is a problem that cannot be overcome even following the guidelines that have been developed by the Clinical Pharmacogenetics Implementation Consortium (https://cpicpgx.org/) to translate genotypes into predicted phenotypes (31).

2.1.1.2. Worldwide Genetic Distribution

It is well documented that most genetic polymorphisms in drug metabolizing enzymes reveal substantial differences in allele frequencies across major human populations. CYP2D6 is no exception, and shows allele frequencies highly varying in world populations, which consequently implies extensive variation in the distribution of the phenotypic response to drugs metabolized by CYP2D6 among worldwide populations.

Previous genetic studies revealed that CYP2D6 diversity was far greater within populations than between groups of populations and that null or low-activity variants occurred at considerably high frequencies in various regions of the world, although specific allelic variants are present at very distinct frequencies among world populations, whereas other are restricted to certain population groups (25).

Furthermore, the macrogeographical spatial patterns of diversity at CYP2D6 revealed to be clinal, and very similar to those shown by neutral markers, indicating that CYP2D6 diversity was modelled by the same factors that randomly affected neutral genomic variation (32). It is known that geography was the main factor influencing genetic differentiation among humans. Both physical distances and geographic barriers limits human communication and interaction, which has led to a global genetic substructure that largely follows geographic clines.

However, populations may be genetically structured, when composed by more than one subpopulation that tend to maintain endogamous practices. These subdivisions, often corresponding to ethnic groups, are not mainly influenced by geography, but instead by social factors, including religion, culture, language, and other sources of group identification, thus leading to varying degrees of genetic structure, depending on the extent of reproductive isolation and endogamy.

From the genetic point of view, the Portuguese represent a homogeneous population that is very well integrated in the context of populations from Western Europe. This scenario does not take into account the Portuguese Roma, who were demonstrated to contribute to some level of population structure in Portugal (33).

3. Portuguese Roma

The Roma, also referred to as Romani and widely known as “Gypsies” among English-speaking people, are a transnational ethnic group, often reputed for their nomadic tradition, that nowadays is mostly spread throughout Europe, Near East, Caucasus and the Americans.

The social structure of the Roma groups is heavily influenced by the traditional endogamy, which is the trend to marry individuals within the same subgroup, since marriages between non-Roma or between Roma from different groups are avoided.

Although their history was largely lost, important insights on the issue have come from scarce historical records documenting the presence in countries where they have lived, from linguistics, and more recently from genetics (34).

Most studies performed so far suggested that the original homeland of the Roma was India, from where they departed around the 5th and 10th century, travelling to Europe, reaching first the Balkans region and later migrating into multiple locations throughout the whole continent in such way that by the 15th century they were already present in Spain and Portugal (34, 35). Genetic and linguistic studies pinpointed affinities between Roma population and various Indian groups. Comparative linguistics studies suggested that North-western Indian languages, such as Punjabi or Kashmiri or Central Indian languages like Hindi are the most related to Romani (35, 36). Genetic studies in groups of European Roma, showed that all groups share high frequency of Y – chromosome lineages, such H1a-M82 and mitochondrial haplogroups like M5a1, M18 and M35b, which are usually absent in European populations, while being rather common in South Asian Populations, especially from Northern India (36, 37). Nevertheless, the ancestral group/geographic region of India from which the Roma groups have originated is still unclear, given the contradictory evidence provided by a recent study that they might descend from southern Indian groups (34, 37).

The Roma populations represent an interesting case of study, especially from a genetic point of view, because due to their endogamous practices, limited gene flow between groups and past history of successive group fragmentations that occurred during their migration into and within Europe, they present signatures of strong genetic drift effects, such as reduced genetic diversity and high linkage disequilibrium (36). Besides, from a pharmacogenetic point of view, the Roma population is still understudied. Although a previous study addressing genes that influence drug response

has already been conducted in the Portuguese Roma Population (38), they still remain very poorly characterized in regard to a fraction of diversity that can have high clinical relevance.

II. AIMS

In the present study we intended to assess, in the Portuguese Roma, genetic diversity in the CYP2D6, a gene very important from a Pharmacogenetic point of view, to evaluate whether this population group show any peculiarity regarding drug response, in comparison with the remaining Portuguese population. In order to achieve this goal, the following partial aims were established:

- Search and selection of SNPs and CNVs with pharmacological relevance and development of PCR and minisequencing multiplex reactions (SNaPshot™);
- Genotyping of a sample of Portuguese Roma population;
- Comparison of the results in the Portuguese Roma with those previously available for the Portuguese host population and other populations, to put the data obtained in a worldwide context.

III. MATERIALS AND METHODS

1. Samples and DNA extraction

In this study, 56 samples from self-identified Portuguese Gypsies were analysed. All samples have been extracted for previous works by the Chelex®-100TM (BioRad) method described in Laureau *et al.* (39).

2. PCR design

We first performed a thorough literature search in order to select SNPs in the CYP2D6 with pharmacogenetic interest. At the end, the following eleven SNPs were chosen: 100 C>T (rs1065852); 1023 C>T (rs28371706); 1659 G>A (rs61736512); 1707 del. T (rs5030655); 1846 G>A (rs3892097); 2549 del. A (rs35742686); 2615 del. AAG (rs5030656); 2850 C>T (rs16947); 2988 G>A (rs28371725); 3183 G>A (rs59421388); 4180 G>C (rs1135840). This set of SNPs allows the identification of the CYP2D6 haplotypes that are widely represented in different human populations. For the sake of simplicity, the CYPD2D6 haplotypes are conventionally referred to as CYPD2D6 alleles (http://www.cypalleles.ki.se/cyp2d6.htm), among which are included some that do not affect enzyme function (i.e. CYP2D6*1, *2, *34, *39), and others that are associated to low or null metabolic activity (i.e. CYP2D6 *3, *4, *5, *6, *9, *10, *17, *41) (23, 25). To obtain genotype data for these SNPs, a SNaPshot™ strategy was applied, as will be described in the next point.

Viewing the detection of cases where CYP2D6 is deleted and the whole gene, primers were designed based on previous studies involving the CYP2D6 gene. For amplification of the whole gene, a pair of primers were designed as described in Sistonen *et al.* (2005) (28) with the exception of the forward primer, which was designed by us since the previous one was located in a highly polymorphic region, resulting in fragment A (Figure 4.c). In order to detect the presence or not of the gene deletion, a pair of primers described in Krammer *et al.* (2009) (40) was selected, resulting in the fragment C (Figure 4.b). Also, a PCR reaction was performed to detect cases where CYP2D6 was duplicated, using a pair of primers identical to the one described in Gaedigk *et al.* (2007) (29), resulting in fragment B (Figure 4.c). Also, in order to access whether there was a gene duplication or not, a control sample from the Human Genome Database Panel – Centre Étude Polymorphism Humain (HGDP-CEPH) was used. Lastly, an approach to amplify the whole duplicated gene in samples positive for the previous approach described was developed, using a set of primers identical to those described in Gaedigk *et al.* (2007) (29), resulting in fragment D (Figure 4.c). Detection of possible non-specific annealing was tested in BLAT (UCSC) (htts://genome.ucsc.edu/cgi-bin/hgBlat?command=start) selecting only the human genome. In order to find out if the primers could form primer-dimer structures and/or hairpins, the Oligocalc program was

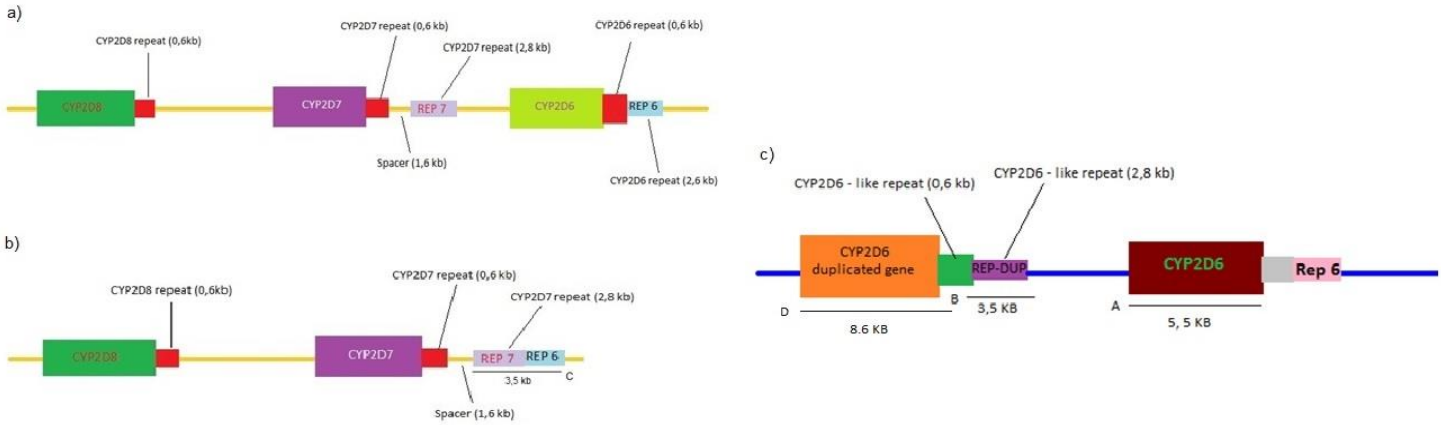

Figure 4 – Schematic representation of different CYP2D6 gene arrangements. a) Normal representation of the CYP2D6 gene cluster; b) Representation of a CYP2D6 gene deletion. As we can notice, comparing with a), the CYP2D6 gene is deleted and a hybrid between the CYP2D6 specific replicated region REP 6 and the specific replicated region of CYP2D7, REP 7, is formed, and using the primers CYP2D7_Seq B1 and CYP2D6_Rep 7R3 (Table 2), we obtain fragment C, indicative of a CYP2D6 gene deletion; c) Representation of duplicated event. Fragment A results from the use of primers CYP2D6_F and CYP2D6_R (Table 2), which were used to assess the presence of the CYP2D6 gene. Fragment B results from the use of primers CYP2D6_DUP_Fw and CYP2D7_DUP_Rv (Table 2), which amplifies a specific replicated region of the duplicated gene, nominated REP-DUP, which is an indicative of a duplication of the CYP2D6 gene. Fragment D is the result of primers CYP2D7_Fw_DUP_TOTAL and CYP2D7_DUP_Rv, amplifying the whole CYP2D6 duplicated gene from samples positive for Fragment B. Adapted from Krammer *et al.* (2009)

used (http://biotools.nubic.northwestern.edu/OligoCalc.html). After the *in silico* evaluation, each pair of primers was tested in individual amplifications in order to assess their functionality. Each one of the primers was used at a final concentration of 4 µM.

Table 2 - Primers used in the PCR amplifications

NAME	Sequence 5' - 3'	SIZE (Kb)
CYP2D6_F	TCAGGAGCTTGGAGTGGGGAGA	5,5
CYP2D6_R	ACTGAGCCCTGGGAGGTAGGTA	
CYP2D7__Seq B1	GTCCCACACCAGGCACCTGTACT	3,5
CYP2D6_Rep 7R3	GAATTAGTGGTGGTGGGTGTTTG	
CYP2D6_DUP_Fw	CCATGGAAGCCCAGGACTGAGC	3,5
CYP2D7_DUP_Rv	CGGCAGTGGTCAGCTAATGAC	
CYP2D6_Fw_DUP_TOTAL	CCAGAAGGCTTTGCAGGCTTCAG	8,6
CYP2D7_DUP_Rv	CGGCAGTGGTCAGCTAATGAC	

The PCR reactions were performed in a final volume of 10 µL, containing 4,5 µL of deionized water, 1 µL of 10x Advantage® Genomic LA Buffer (Clontech), 0,4 µL of Expand Long Range dNTP Pack (Roche Diagnostics GmbH), 1,2 µL of each primer, 0,1 µL of Advantage® Genomic LA Polymerase Mix (Clontech) and 1,6 µL of DNA. The amplification conditions were as follow: 94ºC for 3 minutes, proceeded by 5 cycles at 98ºC for 15 sec, 63ºC for 1.30 min and 68ºC for 8 min, and another 30 cycles at 98ºC for 15 sec, 62ºC for 1.30 min and 68ºC for 8 min and a final extension at 72ºC for 10 min. In the case of the specific region approach and the whole gene duplication approach, the

PCR reactions were performed in a final volume of 10 µL, containing 3,5 µL of deionized water, 1 µL of 10x Advantage® Genomic LA Buffer (Clontech), 0.4 µL of Expand Long Range dNTP Pack (Roche Diagnostics GmbH), 1.2 µL of each primer, 0.1 µL of Advantage® Genomic LA Polymerase Mix (Clontech), 1 µL of Q-solution (Quiagen ®), in order to stabilize the PCR reaction and minimize non-specific amplification, and 1.6 µL of DNA. The amplification conditions for the specific region approach were as follow: 94 ºC for 3 minutes, proceeded by 2 cycles at 98 ºC for 15 sec, 68ºC for 6 min, another 3 cycles at 98 ºC for 15 sec and 66 ºC for 6 min and another 30 cycles at 98 ºC for 15 sec and 64 ºC for 6 min and a final extension at 72 ºC for 10 min. For the whole gene duplication were as follow: 94 ºC for 3 minutes, proceeded by 2 cycles at 98 ºC for 15 sec, 68ºC for 8 min, another 3 cycles at 98 ºC for 15 sec and 66 ºC for 8 min and another 30 cycles at 98 ºC for 15 sec and 64 ºC for 8 min and a final extension at 72 ºC for 10 min.

The amplification reactions were conducted in 2720 Thermal Cycler (Applied Biosystems) or/and T100™ Thermal Cycler (BioRad).

2.1. Electrophoresis

All PCR products were run in agarose electrophoresis (1.5%) in order to assess the success of the amplification reactions and the presence of possible contaminations. Images of the DNA bands in the agarose gel were acquired using the Chemidoc™ XRS+ System, with the aid of Quantity One 1-D Analysis Software.

3. SNP Genotyping

To obtain the genotyping data for SNPs (Figure 5), SNaPshot™ reactions were performed. 11 single base extension (SBE) primers were designed in a similar way for the amplification primers, only excepting that these end right before the target SNPs (Table 3). Primer sequences were identical to those described in Sistonen *et al.* (2005) (28). To promote differences in electrophoretic mobility, a non-annealing tail with different sizes was added to the SBE primers.

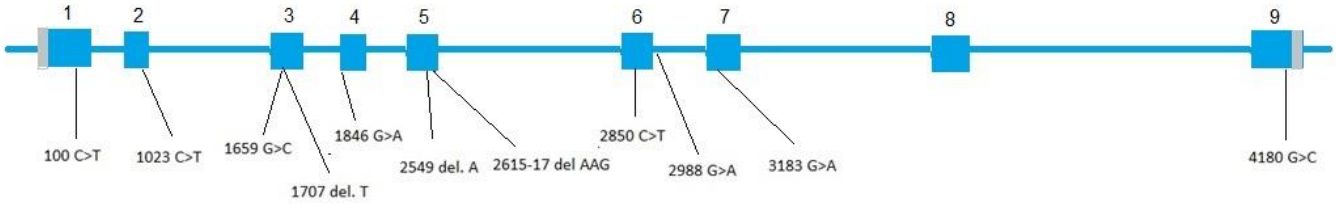

Figure 5 - Representation of the CYP2D6 exons and the different positions of the SNPs analysed. Each number (1-9) represents the position of each exon from 5' to 3'. The primers used of each position are described in table 3. Adapted from Sistonen *et al.* (2005)

Table 3 - Primers used in the SNaPshot™ reactions.

Polymorphism	Rs	Sequence 5´-3'
100C>T	rs1065852	ACGCTGGGCTGCACGCTAC
1023C>T	rs28371706	ACCGCCCGCCTGTGCCCATCA
1659G>A	rs61736512	CTGACAAGCGCGAGCAGAGGCGCTTCTCC
1707delT	rs5030655	GCAAGAAGTCGCTGGAGCAG
1846G>A	rs3892097	CCGCATCTCCCACCCCCA
2549delA	rs35742686	GATGAGCTGCTAACTGAGCAC
2615delAAG	rs5030656	GCCTTCCTGGCAGAGATGGAG
2850C>T	rs16947	AGCTTCAATGATGAGAACCTG
2988G>A	rs28371725	AGTGCAGGGGCCGAGGGAG
3183G>A	rs59421388	TGTCCAACAGGAGATCGACGAC
4180G>C	rs1135840	GTGTCTTTGCTTTCCTGGTGA

Before the SNaPshot™ reactions, a purification step was performed with ExoSAP-IT® in order to remove the surplus of dNTPs and primers present in the PCR product. Each reaction contained 1 µL of amplified product and ExoSAP-IT® and the thermocycler conditions used were 37ºC for 15 min and 85ºC for 15 min. A mix containing all SBE primers was prepared with the following primer quantities: 0,1 µL of the 1659 G>A and 1846 G>A, 0,5 µL from the 4180 G>C and 0,2 µL for the remaining SNPs. The SNaPshot™ reactions were carried out in a final volume of 5,2 µL containing 2 µL of purified PCR product, 2,2 µL of SBE primer mix and 1 µL of SNaPshot™ Multiplex Kit (Applied Biosystems). The reaction conditions were 25 cycles at 96ºC for 10 sec, 50ºC for 5 sec and 60ºC for 30 sec. After this step, the products were treated with 1 µL of SAP enzyme (USB®) at 37ºC for 60 min and 85ºC for 15 min. All these reactions were performed in a 2720 Thermal Cycler (Applied Biosystems) and/or T100™ Thermal Cycler (BioRad). Lastly, 1 µL of the SNaPshot™ purified products were mixed with 12 µL of Hi-Di™ Formamide (Applied Biosystems) and Gene-Scan™ – 120 LIZ™ size standard. Capillary electrophoresis was then carried out in an ABI 3130 Genetic Analyser (Applied Biosystems).

4. Sanger Sequencing

The Sanger Sequencing method was used to confirm results from the SNaPshot™ reactions, using primers described in table 4. Initial purification was carried out on the amplification products as described in section 3. The sequencing reactions were performed in a final volume of 5 µL containing 2 µL of purified amplification product, 1 µL of the amplification primer at 4 µM, 1 µL of BigDye® Terminator v3.1 Cycle Sequencing Kit and 1 µL of sequencing Buffer. The conditions used were as follow: 96ºC for 4 min, followed by 35 cycles at 96ºC for 10 sec, 55ºC for 5 sec and 60ºC for 4 min, and a final step at 60ºC for 10 min. The reactions were carried out in Thermal Cycler (Applied Biosystems) or/and T100™ Thermal Cycler (BioRad). After this, the sequencing products were purified in Sephadex® columns (760 µL) through centrifugation for 4 min at 4400 rpm. The final products were resuspended in 12 µL of Hi-Di™ Formamide and capillary electrophoresis was performed in an ABI 3130 Genetic Analyser (Applied Biosystems).

Table 4 - Primers used in the Sanger Sequencing reactions.

NAME	SEQUENCE 5'-3'	SNPs RANGED	EXPECTED SIZE
CYP2D6_SEQ_1_F	ATCTTCCTGCTCCTGGTGGAC	100C>T	~80 bp
CYP2D6_SEQ_2_F	TTCAAATAGGACTAGGACCTGTA	1023C>T	~250bp
CYP2D6_SEQ_3_R	GAATGTCCTTTCCCAAACCCAT	1659G>A; 1707delT; 1846G>A	~500 bp
CYP2D6_SEQ_4_F	CACAGGAGGGATTGAGACCC	2549delA; 2615delAAG; 2850C>T; 2988G>A; 3183G>A	~800 bp
CYP2D6_SEQ_5_R	TTATTGTACATTAGAGCCTCTGG	4180G>C	~100 bp

5. Data Analysis

The results obtained in the SNaPshot™ reactions were analysed using the GeneMapper® v4.0 software and the sequencing data was analysed with Geneious Pro 5.5.8. The Arlequin Software v3.5 was used to test for Linkage Disequilibrium (L.D.), estimate haplotype frequencies and to test deviations from the Hardy-Weinberg Equilibrium.

In order to perform comparative analysis, data from previous works published was used (25, 30, 41).

To evaluate the significance of the difference between the two proportions, the z-ratio was calculated in the web site http://vassarstats.net/.

IV. RESULTS AND DISCUSSION

1. CYP2D6 Genotyping

After applying the strategy to detect the presence of the entire CYP2D6 gene, positive results were observed in 55 of the 56 individuals tested. In the remaining subject, the suspicion of being homozygous for the deletion was confirmed through the strategy used to identify the deletion of the entire CYP2D6 gene, which was applied for all the samples in this study. In figure 6 is depicted an example of an agarose electrophoresis of different PCR products. As is illustrated, samples positive for the presence of the gene presented a band corresponding to the whole gene (marked arbitrarily as “G”). That band was absent in a unique individual (“X5” in Figure 6), who was also the unique presenting the band expected when submitted to the approach undertaken to test the gene deletion (marked as “X5 D”).

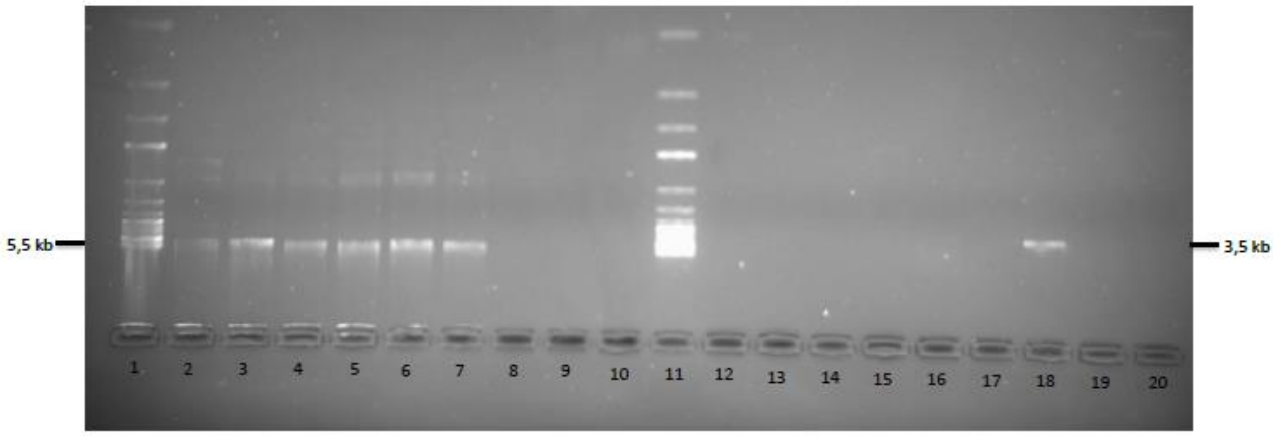

Figure 6 – Example of an Agarose Electrophoresis performed for the analysis of the whole gene and cases of gene deletion. Lanes 1 and 11- DNA molecular weight markers (Ladder 1 kb); Lanes 2 to 9 - samples amplified with the primers designed to detect the whole gene. Lanes 12 to 18 - the same samples amplified with the primers designed to detect deletion of the whole gene. Lanes 10 and 20 – Negative controls of the PCR reactions. Sample in lane 8 doesn't present any band when tested for the presence of the whole gene, but presents a band when tested for the deletion of the whole gene (Lane 18). Sample in lane 9 didn't present any band after the two amplification approaches, due to poor quality of DNA.

The 11-plex SNaPshot™ reaction was successfully developed and applied for all the 56 samples. In figure 7 are shown illustrative CYP2D6 electropherograms obtained for different individuals. The electropherogram at the bottom presented in the figure corresponds to the result observed for sample "X5", identified as a homozygous for the gene deletion, which showed entire failure of the SNaPshot™ reaction, thereby consistent with a gene deletion on this sample.

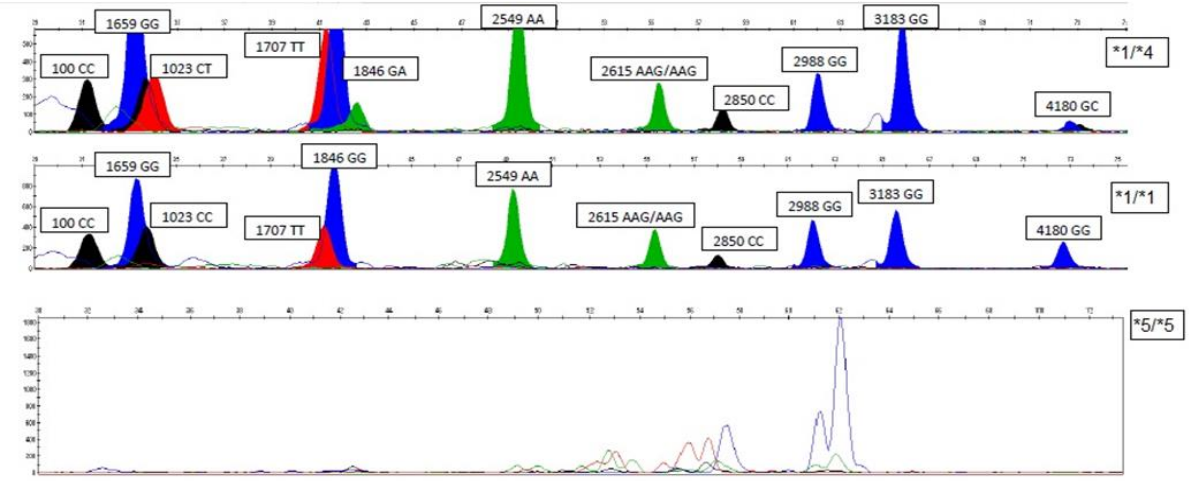

Figure 7 – Example of an electropherogram performed in this study and respective predicted genotype. The last one was obtained from the sample suspected to have a deletion of the whole gene, confirming our suspicion.

Lastly, a molecular strategy directed to the identification of gene duplications was applied to the 55 samples positive for the presence of CYP2D6 (Figure 8), which allowed to identify 3 individuals possibly harbouring that kind of copy number variations. However, when the strategy used to validate the presence of the whole duplicated gene

was applied to these 3 individuals (Figure 9), only in one of them was observed a positive result, which was further confirmed after using the SNaPshot™ technique (Figure 10). Thus, a gene duplication was detected in a unique individual from the studied Roma sample.

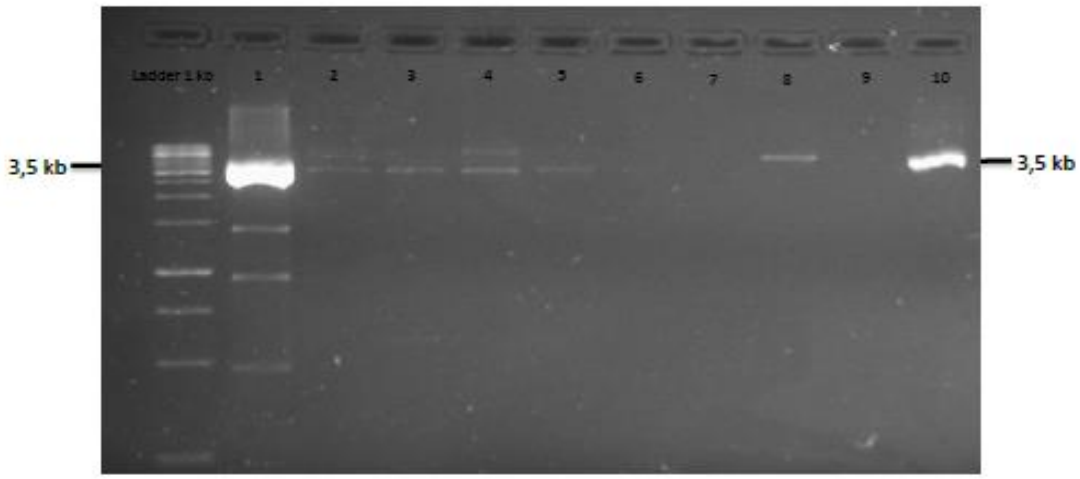

Figure 8 – Example of an agarose electrophoresis performed to determine the presence or not of duplication events. A control sample from the HGDP-CEPH (lane 1) was used as a positive control of the amplification. Sample in lane 10 presents a band identical to the positive control, indicating the presence of a duplication event. The bands presented in the remaining lanes (2-9) are due to non-specific amplification.

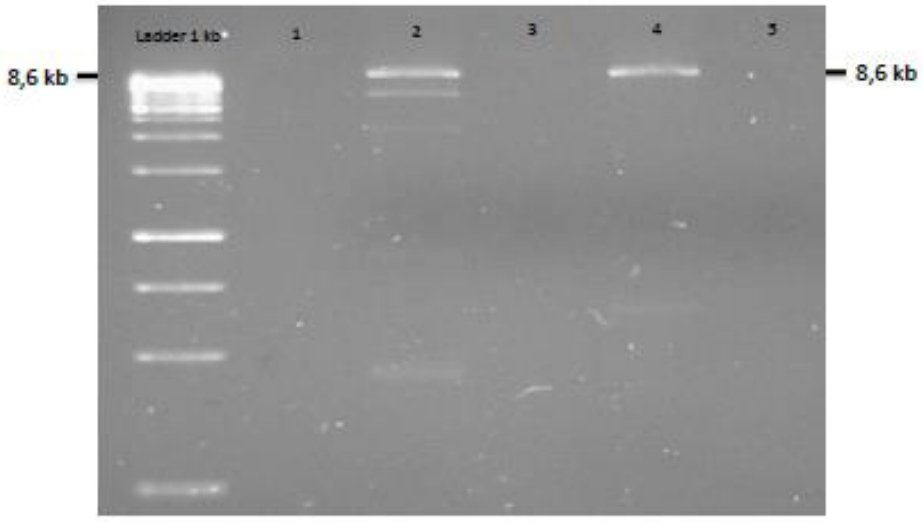

Figure 9 – Example of an agarose electrophoresis performed to determine the presence of the whole duplicated gene. A control sample from the HGDP-CEPH (Lane 2) was used again as a positive control. In lane 1 is the negative control for the amplification. The 3 samples tested (lane 3-5) were those identified through a previous approach as suspected to have a gene duplication. Only one of them (lane 4) presented a positive result in the PCR assay specific for the detection of the whole duplicated gene.

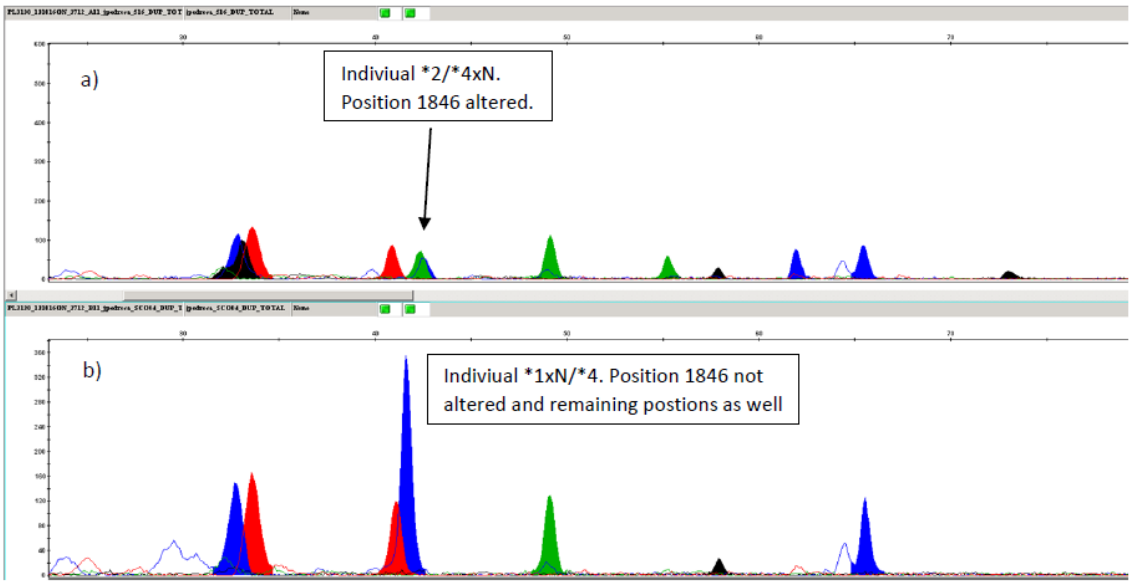

Figure 10 – SNaPshot™ reaction performed to confirm the results of the whole duplicated gene approach. a) Control Sample HGDP00531; b) Sample with duplication identified in the present study. In the electropherogram of the control HGDP00531, known to have the genotype *2/*4N, the position 1846 is altered, presenting an A instead of a G, therefore indicative of the duplication event in the allele *4. In our sample, the electropherogram did not reveal alterations in the position 1846, as well as the remaining positions, therefore indicating that the duplicated gene harbors allele *1.

2. Inference of CYP2D6 diplotypes

Out of the 11 polymorphic positions screened in our sample, no variation was detected at 1023C>T, 1659 G>A, 1707 del, 2615 delAAG and 3183 G>A. For the remaining 6 polymorphisms, tests for Hardy-Weinberg equilibrium were performed

Table 5 – Results from the Hardy – Weinberg equilibrium test obtained for the 11 positions

Position	n	Observable Heterozygosity	Expected Heterozygosity	p - value	s. d.
100 C>T	56	0.48214	0.43356	0.53450	0.00049
1023 C>T	Monomorphic				
1659 G>A	Monomorphic				
1707 delT	Monomorphic				
1846 G>A	56	0.41818	0.40851	1	0
2549 delA	56	0.05455	0.05354	1	0
2615 delAAG	Monomorphic				
2850 C>T	56	0.36364	0.41635	0.34462	0.00048
2988 G>A	56	0.14545	0.13611	1	0
3183 G>A	Monomorphic				
4180 G>C	56	0.29091	0.48440	0.00448	0.00007

(Table 5), which uniquely revealed a significant departure between observed and expected genotypic distributions at 4180 G>C due to an excess of homozygotes (observed heterozygosity: 29,1%; expected: 48,4%), even after the Bonferroni correction for multiple tests was applied.

Table 6 – Results of Linkage Disequilibrium (L.D.) obtained for the 11 positions. Presence of L.D. (+); Absence of L.D. (-)

Positions	100 C>T	1023 C>T	1659 G>A	1707 delT	1846 G>A	2549 delA	2615 delAAG	2850 C>T	2988 G>A	3183 G>A	4180 G>C
100 C>T	*	-	-	-	+	-	-	-	-	-	+
1023 C>T	-	*	-	-	-	-	-	-	-	-	-
1659 G>A	-	-	*	-	-	-	-	-	-	-	-
1707 delT	-	-	-	*	-	-	-	-	-	-	-
1846 G>A	+	-	-	-	*	+	-	+	+	-	+
2549 delA	-	-	-	-	+	*	-	+	+	-	+
2615 delAAG	-	-	-	-	-	-	*	-	-	-	-
2850 C>T	-	-	-	-	+	+	-	*	+	-	+
2988 G>A	-	-	-	-	+	+	-	+	*	-	+
3183 G>A	-	-	-	-	-	-	-	-	-	*	-
4180 G>C	+	-	-	-	+	+	-	+	+	-	*

As expected, significant Linkage Disequilibrium (L.D.) was detected between the intergenic variations examined (Table 6). Accordingly, from the unphased genotypic data, diplotypes were reconstructed for each individual using the EM algorithm implemented in Arlequin, which was also used to estimate frequencies for the inferred haplotypes.

Table 7 - Haplotypes defining different CYP2D6 alleles, corresponding effects in enzyme activity and frequencies in the Portuguese Roma. Note: Haplotype ID corresponds to the identifier provided by Arlequin, while the CYP2D6 allele designations and effects in enzyme activity were assigned according to the CYP Allele Nomenclature Database.

Haplotype I.D	100 C>T	1023 C>T	1659 G>A	1707 del. T	1846 G>A	2549 del. A	2615 del. AAG	2850 C>T	2988 G>A	3183 G>A	4180 G>C	CYP2D6 allele	Enzyme Activity	Frequency
1	T	C	G	T	A	A	AAG	C	G	G	C	*4	None	0,238444 ± 0,0433
2	C	C	G	T	G	A	AAG	C	G	G	C	*39	Normal	0,035977 ± 0,018525
3	T	C	G	T	A	A	AAG	C	G	G	G	*4J	None	0,009322 ± 0,009732
4	C	C	G	T	G	A	AAG	C	G	G	G	*1	Normal	0,317943 ± 0,048101
5	T	C	G	T	G	A	AAG	C	G	G	C	*10	Decreased	0,046051 ± 0,019395
6	C	C	G	T	G	A	AAG	T	G	G	C	*2	Normal	0,179935 ± 0,040293
7	C	C	G	T	G	G	AAG	C	G	G	G	*3	None	0,026786 ± 0,016723
8	C	C	G	T	G	A	AAG	T	A	G	C	*41	Decreased	0,059859 ± 0,025156
9	C	C	G	T	G	A	AAG	C	A	G	G	*41v	Decreased	0,01157 ± 0,010385
10	C	C	G	T	G	A	AAG	T	G	G	G	*34	Normal	0,027237 ± 0,018843
11	C	C	G	T	A	A	AAG	C	G	G	C	*4M	None	0,010337 ± 0,009338
12	T	C	G	T	A	A	AAG	T	G	G	C	*4K	None	0,018683 ± 0,01566
13	-	-	-	-	-	-	-	-	-	-	-	*5	None	0,017857 ± 0,012943

The program has inferred 13 distinct haplotypes in our sample, all of which corresponding to allelic variants or sub-variant already defined in the CYP Allele Nomenclature Database.

In table 7 are presented the detected alleles, their frequencies in the sample of Portuguese Roma and the effect of each allele in enzyme function. Allele CYP2D6*5, which refers to the deletion of the gene and consequently to a non-functional allele, was present in homozygosity in one individual. No heterozygous for the deletion were found, although carriers could be identified with the methodological strategy used to detect this kind of copy number variation. Given that the frequency of CYP2D6*5 was 1,8%, the number of expected heterozygous in a sample of 56 individuals was 1,96, which is a considerably low value, likely explaining that by chance heterozygous were missing in the studied sample of Portuguese Roma.

Four other alleles were detected in the Roma that were associated to absence of enzymatic activity: CYP2D6*4 and its subtypes *4J, *4M and *4K, all bearing the splice site mutation 1846 G>A responsible for loss of activity, and CYP2D6*3, defined by 2549 del.A, which is a frameshift mutation also responsible for a non-functional product. Taken together the alleles conferring none activity summed up 29,8 % in the sample. Two partially functioning variants were identified, CYP2D6*10 and CYP2D6*41, both leading to decreased enzymatic activity, that together were present at 11,7%. The remaining detected alleles were normal function variants, including CYP2D6*1, which was the commonest allele (30,4%), CYP2D6*2, *34 and *39. In total, the normal alleles reached 53.7% in the sample.

As mentioned, the analysis of copy number variation revealed the presence of gene duplications in 1 individual, whose inferred genotype based on the SNaPshot™ results was *1/*4. The approach used to amplify the whole gene duplication also permitted to conclude that the duplicated gene involved the allele *1 of the genotype *1/*4. Nevertheless, the approach does not allow to infer the number of gene copies present in the individual.

3. Metabolic Profiles

In order to predict the theoretical metabolic profiles from the CYP2D6 genotypes, we followed the strategy previously described by Gaedigk *et al.* (25, 31). It is referred to as "Activity Score" System, according to which genotypes are categorized by the ''number of active genes'', alleles are grouped based on their assumed functionality, active scores (AS) are assigned to each genotype and then ASs are translated into a phenotype prediction that include the traditional classification as PM, IM, EM, and UM. To calculate the AS, a value of 1 is assigned to normal alleles (i.e. *1, *2, *34, *39), 0.5 for reduced – activity alleles (i.e. *10, *17), 0 for null variant alleles (i.e. *3, *3xN, *4, *4K, *3xN, *4xN, *5) and 2 for multiplications alleles like *1xN or 2*xN. Therefore, genotypes with AS=0, were considered PM, with AS = 0,5 were considered IM, with AS = 1-2 were considered EM and with AS≥2 were considered UM.

In Table 8 are presented the frequencies of the CYP2D6 genotypes in the Portuguese Roma and their ascribed AS. For the three subjects known to harbour gene duplications, only one presented a real duplicated event, which was the individual with the genotype *1/*4, and the duplication event was in allele *1, but we still cannot infer the extent of the duplication. Because he had at least two active gene, it is possible to deduce that the active score is two or greater than two. In terms of predicted metabolic profiles, the categories poor and intermediate metabolizers can be excluded, but he might fall either in the group of extensive or ultra metabolizers, depending on the extent of the duplicated allele.

Table 8 - Frequencies of CYP2D6 genotypes in the Portuguese Roma population. Concerning the genotype referred to as *1xN/*4, since it was not possible to infer the number of gene copies, while knowing that the duplicated gene involved allele *1, its number of active genes and active score is at least 2 although it can be greater than 2. N – Duplication event

Genotype	n	Active Genes	Active Score	Frequency
*1/*1	7	2	2	0,1250
*1/*2	3	2	2	0,0536
*1/*3	3	1	1	0,0536
*1/*4	9	1	1	0,1607
*1/*4J	1	1	1	0,0179
*1/*34	1	2	2	0,0179
*1/*41	4	2	1,5	0,0714
*2/*2	3	2	2	0,0536
*2/*4	6	1	1	0,1071
*2/*10	3	2	1,5	0,0536
*2/*39	1	2	2	0,0179
*2/*41	1	2	1,5	0,0179
*4/*4	2	0	0	0,0357
*4/*4M	1	0	0	0,0179
*4K/*4K	1	0	0	0,0179
*4/*10	1	1	0,5	0,0179
*4/*39	3	1	1	0,0536
*4/*41	2	1	0,5	0,0357
*5/*5	1	0	0	0,0179
*10/*41	1	2	1	0,0179
*34/*34	1	2	2	0,0179
*1xN/*4	1	≥ 2	≥ 2	0,0179

Taking into account the ascribed ASs, the great majority of the Portuguese Roma were predicted to be extensive (83,9%) or intermediate metabolizers (5,4%) (Figure 11). However, a substantial proportion of 8,9% (Figure 11) was assigned to the category of poor metabolizers, giving thus a prediction of the proportion of Roma at high risk of experiencing adverse reactions when treated with drugs that are metabolized by CYP2D6.

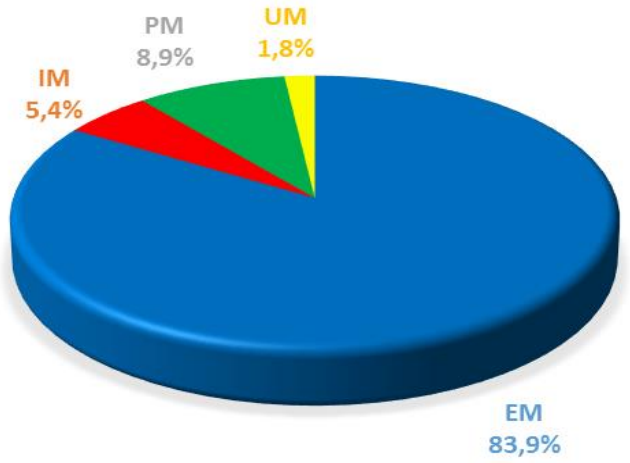

Figure 11 - Frequencies of theoretical metabolic profiles in the Portuguese Roma population. EM – Extensive Metabolizers; IM – Intermediate Metabolizers; PM – Poor Metabolizers; UM – Ultrarapid Metabolizers.

As stated before, while we could infer that only one individual presented a duplication, it was not possible to deduce the extent of the duplication event, which is the total of gene copies contained in the chromosome with the duplication. This individual has a minimum number of two CYP2D6 genes but in fact can have more than two copies, and so he is the unique candidate in the Roma sample that theoretically can be ultrarapid metabolizer. As follows, we can only anticipate that this metabolic category in the Portuguese Roma might range from 0 and the maximum frequency of 1,8%. Depending on that, the frequency of extensive metabolisers can also vary between the minimum estimate at 83.9% and a maximum of 85.7%, with the latter value registered if the individual is not ultra metabolizer.

4. Comparison with other populations

To put the data here obtained for the Portuguese Roma in a broad population context, we took advantage on a recently study that constitutes the most comprehensive work up to now performed summarizing CYP2D6 allele frequencies, diplotypes and predicted phenotype across major populations (25). The study was based on an exhaustive data compilation from the literature, which ended up with hundred entries for multiple ethnic groups and populations from different geographical regions, including two about the Portuguese previously studied for CYP2D6 (30, 41).

Among the many population entries in the work of Gaedigk *et al.* (2016), we selected a few to illustrate the distribution of CYP2D6 alleles in European and Asian populations, which are presented in Table 9, where are also included the results from the present study.

As we can notice, there are many alleles without information for most of the populations listed in Table 9. This is not a consequence of any bias arising from our selection criteria, but only the reflex of the variety of genotyping strategies used in different studies. Owing to the major contribution to the metabolism and biotransformation of numerous drugs, CYP2D6 is one of the pharmacogenes most extensively analysed, although based in genotyping assays that greatly varied between studies. Many examined a small number of variations within the gene, usually coinciding with the allelic variants most common and with a widespread distribution, and consequently several variants certainly escaped detection. This causes a problematic skewed distribution, falsely overestimating the frequency of the default allele, that is the allelic category in which are included all those alleles not bearing any of the variations examined.

Table 9 – Frequencies of CYP2D6 variant alleles in different populations. Regarding the results of Correia *et al.* (2009), since they don't indicate the frequency of normal alleles, for comparative analysis, we decided to attribute the remaining frequency left (0,745) as normal alleles n – Population Size; Sum – Summatory of frequencies of each category

Population	n	Normal Alleles						Defective Alleles						Reduced - Activity Alleles					Multiplications
		*1	*2	*34	*39	Others	Sum	*3	*4	*5	*6	Others	Sum	*9	*10	*17	*41	Sum	
Portuguese Roma	56	0,318	0,180	0,027	0,036	-	0,561	0,027	0,277	0,018	0,000	-	0,321	0,000	0,046	0,000	0,060	0,106	0,018
Portuguese	100	-	-	-	-	0,745	0,745	0,014	0,133	0,028	0,019	-	0,194	-	-	-	-	0,000	0,061
Portuguese	300	0,377	0,333	-	-	-	0,710	0,005	0,182	0,025	0,002	-	0,214	-	0,035	0,01	-	0,045	0,032
Spanish	105	0,320	0,405	-	-	-	0,725	0,010	0,138	0,033	0,0095	-	0,190	0,0238	0,019	-	-	0,043	0,043
Macedonians	184	0,185	0,108	0,054	0,146	0,119	0,612	0,008	0,187	0,091	0	-	0,286	0,016	0,027	-		0,043	0,059
Hungarians	112	0,404	0,151	-	-	0,085	0,640	0,018	0,204	0,018	0,004	0,004	0,248	0,014	-	-	0,08	0,094	0,018
Hungarian Roma	426	0,580	-	-	-	-	0,580	-	0,204	-	-	-	0,204	-	0,216	-	-	0,216	-
Turkish	404	0,371	0,353	-	-	-	0,724	0	0,113	0,015	0,007	0,005	0,140	0,006	0,061	0,011	-	0,078	0,058
Russian	1230	0,351	0,357	-	-	-	0,708	0,013	0,177	0,016	0,01	0,004	0,220	-	-	-	0,076	0,076	-
Austrians	93	0,362	0,273	-	-	-	0,635	0,005	0,14	0,016	0,005	-	0,166	0,016	0,043	0	0,124	0,183	0,016
Germans	589	0,364	0,324	-	-	-	0,688	0,02	0,207	0,020	0,009	0,008	0,264	0,018	0,015	-	-	0,033	0,019
Danish	244	0,386	0,184	-	-	-	0,570	0,016	0,197	0,053	0,013	-	0,279	0,029	0,016	0	0,098	0,143	0,008
Swedish	281	0,374	0,324	-	-	-	0,698	0,014	0,244	0,043	0,0009	-	0,302	-	-	-	-	0,000	-
Finnish	86	0,36	0,384	-	-	-	0,744	0,017	0,081	0,035	0,012	-	0,145	0	0,012	0	0,058	0,070	0,041
North Indians	125	0,396	0,336	-	-	-	0,732	-	0,1	-	-	-	0,100	-	0,168	-	-	0,168	-
Southern Indians	447	0,458	0,348	-	-	-	0,806	0	0,073	0,019	-	-	0,092	-	0,102	0	-	0,102	-
West Indians	160	0,435	0,231	-	-	-	0,666	0,003	0,103	0,019	0	-	0,125	0	0,059	0	0,125	0,184	0,025
Iranians	100	0,435	0,32	-	-	-	0,755	-	0,125	0,03	-	-	0,155	-	0,09	0	-	0,090	-
Bedouins	50	0,49	0,09	-	-	-	0,580	0	0,04	0,03	-	-	0,070	-	0,02	0,02	0,29	0,330	0,02

The default allele is usually assumed to be CYP2D6*1 also referred to as the “wild-type” allele, which might then encompass the “non-normal” alleles that could not be detected with the screened positions. In this work we used a CYP2D6 genotyping technique that allows the detection of 11 of the most relevant polymorphic positions in the gene covering the majority of the clinically important mutations (28). However, most of those positions were not included in previous conventional typing methods, not including those more recently developed or obviously those relying in high-throughput assays that are still very scarce (42, 43). This represented a setback regarding comparative statistical analysis. For instance, allele *41, which is a reduced - activity allele, is characterized by the presence of A instead of a G in position 2988, but also presents a T instead of a C in position 2850 and a C instead of a G in position 4180. Allele *2 is characterized by the presence of a T instead of a C in position 2850 and a C instead of a G in position 4180. If the SNP 2988 G>A is not analysed, we can misidentify an individual as *2/*2, when he could be *2/*41 or *41/*41.

Another issue in the comparative analysis was the difficulty to deal with multiplications. Although we developed a strategy to identify the allele duplicated, we could not infer the number of copies present with this approach. Therefore, and for the sake of simplicity, we have agglomerated the distinct types of multiplications reported in the populations considered in Table 9 in one single group, named “Multiplications”.

Regarding the set of normal alleles as a whole, including *1, *2, *34, *39 and “Others”, we can see that the Portuguese Roma present one of the lowest frequencies among Eurasian populations. For instance, comparatively to the host population, normal alleles summed up 71%, whereas the frequency in the Roma was substantially lower (56,1%), with the difference being statistically significant (P= 0,001 in the z-ratio for the significance of the difference between the two proportions). In compensation, the total frequency of defective alleles in the Portuguese Roma (32,1%) lies in the limit of the upper range of values until now reported. Focusing again in the comparison with the Portuguese host population, all defective alleles present in the Roma (*3, *4 and *5) are also present in the Portuguese host population. However, it is remarkable the increased frequency of the null allele CYP2D6*4 in the Roma (27,7%), comparatively to other Portuguese, among whom the frequency is considered below 20%.

As for reduced – activity alleles, two variants are present in the Portuguese Roma: CYP2D6*10, with a frequency of 4,6% and CYP2D6*41, with a frequency of 7,1%. The first was also found in the Portuguese host population, while the second one was not screened up to now in other Portuguese. Both CYP2D6*10 and *41 are encountered in most other European populations, with a typical frequency range in which fall the values observed in the Portuguese Roma. Interestingly, CYP2D6*10 is very well represented in Indian populations, particularly from the North where it reaches 16.8%, as well is CYP2D6*41, at least in West India (12,5%), once it was not yet screened in other Indian populations. Actually, the distribution of latter allele is still very scarcely known because often it was not included in the CYP2D6 typing methods. The highest frequency up to now reported in Europe was among Austrians, attaining 12.4%, thus a value similar to the reported for Western Indians.

Data for CYP2D6 was available for a Roma group from Hungary (Table 9), although based on a panel of variations with low resolution, and so only allowed to identify the alleles CYP2D6*4 and *10, two variants we found in the Portuguese Roma, but also widespread in other populations. However, a finding that deserves attention is the high frequency of CYP2D6*4 in the Hungarian Roma (22,5%), though lower than among the Portuguese Roma (27,7%) as well as the very high proportion of CYP2D6*10 (26.6%). In non-Roma European populations, very rarely CYP2D6*4 exceeds 20% (two unique reports in the Swedish, 24.4%, and, suggestively, in the Hungarian non-Roma, 20.4%), while the values of CYP2D6*10 in Europe are usually lower than 5%. The atypically increased frequencies of some CYP2D6 alleles in the Portuguese and Hungarian Roma, is fully consistent with what is known about the demographic history of Roma groups, which along time was always accompanied by remarkable founder events.

In respect to the multiplications, we can see that the frequency of 1.8% in the Portuguese fits well the values reported for other populations. Notably, the distribution of multiplications across populations is quite uniform.

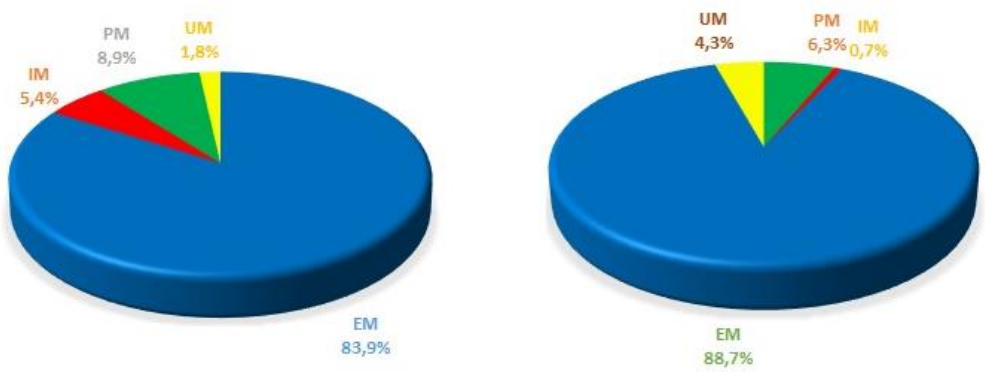

Figure 12 - Frequencies of theoretical metabolic profiles. a) Portuguese Roma (current study); b) Portuguese host (Albuquerque *et al.* 2013). EM – Extensive Metabolizers; IM – Intermediate Metabolizers; PM – Poor Metabolizers; UM – Ultrarapid Metabolizers;

In terms of metabolic profiles, we recall that the inferred theoretical categorization in the Portuguese Roma was 83.9% for EM, 5.4% for IM, 8.9% for PM and 1.8% still partially unknown, which can only fall in EM or UM, but for the sake of comparative analysis, we decided to consider it UM. In order to compare this profile with that from the Portuguese non-Roma, we applied the same criteria to the raw data presented by Albuquerque *et al*, which has yielded the following proportions: 88.7% for EM, 0.7% for IM, 6.3% for PMs and 4.3% for UM. Figure 12 shows the profiles of the Portuguese Roma and non-Roma, which despite not differing too much, reveal that the proportion of PMs in the Roma is slightly higher than in non-Roma, but the difference is not statistically significant (P=0.228), and that the proportion of IM in the Roma is also higher than in non-Roma, but in this category difference reaches statistical significance (P=0.006). This is due to elevated frequency of null variant alleles detected in the Portuguese Roma, namely alleles *4 and *10, which have contributed to diminish the frequency of EMs at the cost of increased frequency of PMs plus IMs individuals.

V. FINAL REMARKS

In this study we have applied a high resolution SNaPshot™ methodology to characterize CYP2D6 in a sample of Portuguese Roma.

The results obtained led to conclude that when Portuguese Roma are submitted to treatments with drugs in which CYP2D6 acts in the biotransformation, they likely will be more susceptible to adverse drug reactions than other Portuguese, because they have higher frequencies of null CYP2D6 alleles. And this is where, like it has been discussed throughout the years, pharmacogenetic will have a preponderant role in clinical practice. With the improvement and introduction of genetic test in the clinical setting, there will be the possibility of developing the so called “Personalized Medicine”, which will give the physician the tools needed to not only prescribe the right dose of the drug necessary, but also monitor the response of the patient throughout the treatment. Expectation are growing with the raise of high-throughput genomic approaches. Respecting CYP2D6 micro-chip/array analysis is already often applied, it has some disadvantages because this methodology cannot discriminate accurately the number of active genes, which is critical in cases of UM individuals (30). Next generation sequencing is a powerful tool, but was also demonstrated to be problematic in the analyses of CYP genes. Therefore, there is an imperative need to develop a more reliable, efficient and cost – effective method, in order to be more commonly used in clinical practice. There has been some works developed in that way, mainly through the use of WGS (Whole – Genome Sequencing). This method has been successfully applied to the molecular diagnosis of genetic diseases, particularly in a clinical paediatric context, in cases of neurodevelopment disorders, to diagnose suspected underlying genetic diseases (43). Although we are still far of recommending WGS as the platform for routine pharmacogenetic testing, a study was already published by Twist *et al.* (42), reporting the development of a “computational method for automated derivation of diploid functional haplotypes from unphased WGS” for the CYP2D6 gene, designated as “Constellation”, which allows to detect all gene variants, including gene conversions and rearrangements. Nevertheless, the method encountered some setbacks as, for instance, it cannot consistently detect some rare variants like CYP2D6*68+*4, a tandem arrangement featuring a hybrid CYP2D6 / CYP2D7 gene upstream of a nonfunctional

CYP2D6*4. Despite the many works and efforts towards the implementation of pharmacogenetics in clinical practice, its application is still somehow new.

Another issue that needs further investigation is the correlation between the predicted metabolic profiles and levels of enzymatic activity assessed through biochemical assays. Although strong correlations have been reported mainly for European populations, given the complexity of the variations in CYP2D6, more studies are still needed to validate inferences based on genotypic data and avoid thus misidentification of metabolic categories. For instance, an individual predicted to be EM, might have an unsuspected and so unscreened null variant allele causing decrease of enzyme activity, which would escape detection by conventional genotypic analysis.

With the development of this study, even though our primary goal was to explore the knowledge regarding allelic frequencies and theoretical metabolic profiles at the CYP2D6 gene in the Portuguese Roma, we also intend to aware to the impact that pharmacogenetics can have in clinical practice and that there is a great need to improve on this field. The implementation of Pharmacogenetics in the clinical setting cannot exempt the individualization of genetic tests. However, the knowledge of the specificities of groups like the Roma might alert for the need to rethink dedicated measures in terms of health care.

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