Universidade de Lisboa
Faculdade de Farmácia

Prevalence of *CYP2D6* and *CYP2C19* genotypes and metabolizer status in patients on neuropsychiatric therapy

Application to adverse drug reactions investigation

Ana Sofia Sequeira Pereira

Dissertation supervised by Doctor Maria Luís Cardoso, Senior Pharmacist and co-supervised by Professor Doctor Isabel Maria Antolin Rivera, Assistant Professor with Habilitation.

Master course in Biopharmaceutical Sciences

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The studies presented in this thesis were performed in the Department of Health Promotion and Non Communicable Diseases Prevention at National Institute of Health Doctor Ricardo Jorge (INSA), under the supervision of Doctor Maria Luís Cardoso, PhD, Senior Pharmacist and the co-supervision of Professor Isabel Rivera, PhD with Habilitation. This work is integrated in the project Pharmacogenetics in Neuropsychiatric Diseases, Oncology and Organ Transplantation.

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RESUMO

A identificação e caracterização de polimorfismos genéticos em genes envolvidos na farmacocinética e/ou na farmacodinâmica pode proporcionar um conhecimento profundo sobre as diferenças inter-individuais na resposta aos fármacos, permitindo otimizar a seleção dos medicamentos e o ajuste da dose, para uma melhoria da eficácia do tratamento e minimizando a ocorrência de reações adversas.

Neste estudo, recorremos a várias metodologias (MLPA; PCR-longo e sequenciação de Sanger) para detetar variantes nos genes *CYP2C19* e *CYP2D6,* caracterizar o seu genótipo e inferir o fenótipo num grupo de indivíduos portugueses com terapia antidepressiva e/ou antipsicótica. Estes dois genes codificam para enzimas importantes no metabolismo dos referidos fármacos.

O estudo do gene *CYP2C19* revelou 44,64% de *metabolizadores normais*, 4,46% *metabolizadores ultra-rápidos*, 27,68% *metabolizadores rápidos*, 22,32% *metabolizado-res intermédios* e cerca de 1% de metabolizadores *lentos.*

Relativamente ao *CYP2D6*, o estudo revelou que 55,36% da população em estudo são *metabolizadores normais*, 12,50% são *metabolizadores intermédios*, cerca de 1% são *metabolizadores lentos* e igual percentagem são *metabolizadores ultra-rápidos*. Em 30,36% da população em estudo, não foi possível determinar o fenótipo.

Daqui se infere que pelo menos 7% da população apresentam fenótipos extremos que implicariam optar, caso se dispusesse dos dados de farmacogenética antes do início do tratamento, por uma medicação alternativa cujas vias de destoxificação não envolvessem os genes em estudo; além disso, em mais de 50% da população estudada haveria espaço para melhoria da terapêutica com base no ajuste da dose.

Permanecem dificuldades em caracterizar certos haplótipos e híbridos mais complexos com as metodologias de genética molecular mais comuns, sendo a sequenciação por nanoporo uma abordagem com potencial de solucionar o problema visto permitir, numa só corrida, fazer sequenciar, identificar variantes pontuais, e rearranjos de elevada complexidade e simultaneamente haplotipar.

PALAVRAS-CHAVE

Genotipagem; farmacogenómica; *CYP2D6*; *CYP2C19*; medicamentos usados em psiquiatria

ABSTRACT

The identification and characterization of genetic polymorphisms in genes involved in pharmacokinetics and/or pharmacodynamics can provide in-depth knowledge about inter-individual differences in response to drugs, allowing optimization of drug selection and dose adjustment, for improvement on treatment efficacy and minimizing adverse drug reactions.

In this study, we used several methodologies (MLPA; long-PCR and Sanger sequencing) to detect variants in *CYP2C19* and *CYP2D6* genes, to characterize their genotype and infer the phenotype in a group of Portuguese patients on antidepressant and/or antipsychotic therapy. These genes code for important enzymes involved in the metabolism of these drugs.

The *CYP2C19* gene study revealed 44.64% *normal metabolizers*, 4.46% *ultra-rapid metabolizers*, 27.68% *rapid metabolizers*, 22.32% *intermediate metabolizers* and about 1% of *poor metabolizers*.

For *CYP2D6*, the study revealed that 55.36% of the individuals are *normal metabolizers*, 12.50% are *intermediate metabolizers*, about 1% are *poor metabolizers* and an equal percentage are *ultra-rapid metabolizers*. In 30.36% of the study population, the phenotype could not be determined.

This suggests that at least 7% of the population have extreme phenotypes that would require, if pharmacogenetic data were available preemptively, an alternative medication whose detoxification pathways do not involve these two genes. Further, in more than 50% of the studied population there would be opportunity for therapeutic improvement based on dose adjustment.

Difficulties remain in the characterization of more complex haplotypes and hybrids with common molecular genetic methodologies, therefore nanopore sequencing is an approach that has the potential to solve this problem, as it allows, in a single run, sequencing, identification of variants, and highly complex rearrangements, as well as haplotyping.

KEYWORDS

Genotyping; pharmacogenomics; *CYP2D6; CYP2C19*; neuropsychiatric drugs

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ABBREVIATIONS

ADR – Adverse Drug Reaction

ATC – Anatomical Therapeutic Chemical Code

bp – base pair

CGH – Comparative Genomic Hybridization

CNS – Central Nervous System

CNV – Copy Number Variation

CPIC – Clinical Pharmacogenetics Implementation Consortium

CYP – Cytochrome P450

DGS – Direção Geral da Saúde

DPWG – Dutch Pharmacogenetics Working Group

EC – European Commission

EMA – European Medicines Agency

EU – European Union

FDA – Food and Drug Administration

IM – Intermediate Metabolizers

INSA – Instituto Nacional de Saúde Doutor Ricardo Jorge

Kb – Kilobase

MLPA – Multiplex Ligation-dependent Probe Amplification

MS – Member States

NM – Normal Metabolizers

OCDE – Organização para a Cooperação e Desenvolvimento Econômico

PCR – Polymerase Chain Reaction

PGx – Pharmacogenomics

PharmGKB – Pharmacogenomics Knowledgebase

PM – Poor Metabolizers

PPI – Proton Pump Inhibitors

PRAC – Pharmacovigilance Risk Assessment Committee

qPCR – Quantitative real-time Polymerase Chain Reaction

SNP – Single Nucleotide Polymorphism

SNV – Single Nucleotide Variant

SSRI – Selective Serotonin Reuptake Inhibitors

TCA – Tricyclic Antidepressants

UM – Ultra-rapid Metabolizers

UTI – *Unidade de Tecnologia e Inovação*

VIP – Very Important Pharmacogenes

WBC – White Blood Cells

WHO – World Health Organization

WT – Wild Type

XL- PCR - Long-Range PCR

1. INTRODUCTION

1.1. Mental disorders

Mental disorders are one of the most common diseases worldwide, which are ranked as the third leading cause of global disease burden (Noori et al., 2018). Portugal is positioned as one of the countries with the highest prevalence of mental disorders among European countries and among the member states of the Organization for Economic Cooperation and Development (OCDE) (Concelho Nacional de Saúde, 2019).

Over the years the topic of mental health has been more addressed, especially since the beginning of COVID-19 pandemic due to the occurrence of increased cases of mental disturbances such as depression, anxiety, insomnia, and stress, and consequently the intake of neuropsychiatric medication has also increased (Hossain et al., 2020; Jones et al., 2021; Samji et al., 2022).

During the first year of the COVID-19 pandemic, the global prevalence of anxiety and depression increased by 25%, according to a scientific summary released by the World Health Organization (WHO) (WHO, 2022).

In Portugal, a study from Institute of Public Health at the University of Porto collected information between November 2020 and February 2021 from a set of 929 individuals over age 18 and residing in Portugal. The study showed that 26.9% of the subjects had symptoms of anxiety, 7% of depression, and 20.4% had symptoms of both disorders, especially after the start of the pandemic (Aguiar et al., 2022).

1.2. Neuropsychiatric drugs

The pharmacological treatment of these mental diseases is essentially supported by neuropsychiatric drugs, which are compounds that target areas of the Central Nervous System (CNS), decreasing or suppressing the symptoms caused by these disorders (Wong et al., 2011).

Neuropsychiatric drugs are used not only to treat a wide variety of neurological conditions and psychiatric disorders, but also have other applications, such as anesthetics in surgical procedures (Sathyanarayana and Andrade, 2016; Belinskaia et al., 2019).

The pharmacological action of these drugs depends on several factors, not only the effect on the neuron but also all the system as a complex circuit of interconnected neurons and glia cells. Due to this complexity, the neurobiological mechanism of neuropsychiatric drugs is not well reflected on the current classification or chemical similarity but may be better captured by molecular drug-target interactions such as specific metabolizing enzymes, mainly Cytochrome P450 enzymes (the most involved being CYP2C19 and CYP2D6) (Noori et al., 2018).

According to the pharmacotherapeutic classification of medicines, approved by *Despacho No. 4742/2014* of the Minister of Health, which approves the pharmacotherapeutic classification of clinical drugs in Portugal, medicines known as psychotropic drugs are the subgroup 9 the pharmacotherapeutic group 2 (medicines for the CNS). Among psychotropic drugs, four classes can be distinguished: 1 - Anxiolytics, sedatives and hypnotics; 2 - Antipsychotics; 3 - Antidepressants; 4 - Lithium.

In accordance with the Anatomical Therapeutic Chemical Code (ATC) classification system (https://www.whocc.no/):

- Antipsychotics have the code N05A. That means that they are in the pharmacotherapeutic group associated with the nervous system (N), and in the subgroup of psycholeptics (05) with designation A. These drugs are divided into the subgroups of typical (for example haloperidol, flufenazina and cloropromazina) and atypical (for example clozapine, quetiapine and risperidona).

- Anxiolytics, sedatives and hypnotics have the code N05B and N05C. Again, they are found in the pharmacotherapeutic group associated with the Nervous System (N), but belong to the subgroup of psycholeptics (05) with the designation B and C. There are three types (i) barbiturates, (ii) benzodiazepines which are the most used, and (iii) a miscellaneous of anxiolytics, sedatives and hypnotics group.

- Antidepressants have the code N06A. They are part of the subgroup of psychoanaleptic drugs (06) with designation A. Antidepressants are mainly classified as: Tricyclic antidepressants (TACs), Selective Serotonin Reuptake Inhibitors (SSRIs), Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs) and Monoamine-Oxidase Inhibitors (iMAO).

- Lithium has the code N05AN01 and it is one of the first-line drugs for the treatment of bipolar disorder.

1.3. Adverse Drug Reactions (ADRs)

In the general population, one of the body systems most affected by adverse drug reactions (ADRs) is the CNS, resulting in effects on the individual's cognition and motor functions due to poor drug choice and dosage (Khalil and Huang, 2020).

ADRs are according to the World Health Organization (WHO) "a response to a drug that is noxious and unintended, and which occurs at doses normally used in man" (WHO, 2002). However, the lack of effectivity has also been considered an adverse event.

ADRs show several levels of severity. The most severe situations are life threatening, require hospitalization or prolongation of existing hospitalization, or result in the persistence of significant disability or dead (WHO, 2002).

In order to avoid ADRs, the field of pharmacogenomics (PGx) studies the genetic contribution to interindividual variability in drug response, particularly genes and variants that affect pharmacokinetics or pharmacodynamics, contributing to these effects and lack of efficacy (Nicholson et al., 2021).

The pharmacological effects of a drug depend on the patient's dose-response relationship. The size of the effect and the doses that cause benefit and harm will differ among individuals. Some individuals are more likely than others to suffer harm at any given dose. Thus, susceptibility to ADRs can occur by influenced off genetic or non-genetic factors (Camacho et al., 2020; Osanlou et al., 2018).

1.3.1. Genetic factors

Genetic makeup determines the pharmacokinetics and pharmacodynamics of drugs. This is because genes encode proteins, such as metabolizing enzymes and receptors, playing an important role in the absorption, distribution, detoxification, and excretion of clinical drugs. Thus, modifications in genes play a role in the response to the drug since it can affect how the drug works (efficacy) or affect whether the drug causes adverse events (toxicity). This fine-tuning of efficacy and toxicity is the main goal of PGx implementation in healthcare (Weinshilboum and Wang, 2017; Tkachenko and Dinges, 2018).

Individual differences among patients can lead to unexpectedly higher or lower blood drug concentrations when recommended therapeutic doses are used. In turn, low

blood drug levels decrease therapeutic efficacy, whereas higher blood and cellular levels increase toxicity, and ADRs are more likely to occur (Osanlou et al., 2018; Wake et al., 2019).

Modifications in genes can occur in one or more nucleotides of the DNA sequence, by insertion, deletion, or substitution. This alterations in a single nucleotide are the most common genetic variants and universally present in the human genome and they are called Single Nucleotide Variant (SNV), if the population frequency is >1%, this modification is referred to as Single Nucleotide Polymorphism (SNP). Nevertheless, larger variations such as complete gene deletions, rearrangements, or copy number variations (CNVs) may also occur (Rodríguez-Vicente et al., 2016; Roden et al., 2019).

CNVs are unbalanced structural rearrangements of the genome of at least 1 Kb in length, that lead to differences in the number of copies of particular DNA sequences among individuals of the same species (Ionita-Laza et al., 2008; Pös et al., 2021). It has been estimated that up to 12% of the human genome displays CNVs, thus contributing to population diversity and evolutionary processes (Pös et al., 2021). Furthermore, CNVs play an important role in a large number of imbalances that alter the diploid state of a locus, so that copy numbers can increase (duplications or multiplications) or decrease (deletions) (Carson et al., 2006; Li and Olivier, 2013). Both common and rare CNVs have been associated with genetic susceptibility for many diseases including neurodevelopmental and neurodegenerative disorders, hematological and cardiovascular diseases, and even for cancer. Thus, CNVs may be used as disease biomarkers and their identification is relevant for clinical human genetics.

1.3.2. Non-genetic factors

There are also other non-genetic factors that influence ADRs (Ferner and Aronson, 2019):

Age - some ADRs are more common in infants and children who have immature physiological systems; others are more frequent in the elderly mainly due to failing physiological systems, co- morbidities and polymedication;

Sex - some ADRs are more prevalent or even limited to one sex for biological reasons;

Physiological changes – pregnancy, menstrual cycle and circadian rhythm influence ADRs;

Exogenous factors - environmental factors (e.g. sun exposition), food, dietary supplements, can also influence ADRs;

Disease - diseases can affect drug absorption, distribution, metabolism, and elimination.

1.3.3. Adverse Drug Reactions Monitoring

ADRs are a major problem throughout new drugs development process. According to WHO, no drug is completely risk-free, so it is essential to permanently monitor its safety to ensure that, throughout its life cycle, the benefits of each drug outweigh the risks of its use. In this way, we have Pharmacovigilance, which is the "science and activities related to the detection, assessment, understanding and prevention of adverse effects or any other drug-related problem".

In the European Union (EU) there is a regulatory network composed of the competent authorities of each member state (MS), the European Commission (EC), and the European Medicines Agency (EMA), which is responsible for granting marketing authorizations and for the supervision of medicinal products, including the area of pharmacovigilance. EMA is responsible for the coordination of all activities within this network (EMA, 2021)

Internationally, EMA works closely with the United States Food and Drug Administration (FDA), which shares information on drug safety issues and anticipate regulatory actions, public information and communication prior to decision making and publication, and with WHO, which reports any actions taken on centrally authorized medicines that may have a bearing on public health protection in countries outside the EU (INFARMED, 2018; EMA, 2021).

As a drug starts being marketed, health professionals should be aware and attentive, having the duty to report suspected ADRs through their National Pharmacovigilance Systems; in the case of Portugal is INFARMED - Autoridade Nacional do Medicamento e Produtos de Saúde, I. P., which evaluates ADRs according to a standardized criteria and then sends the information to the European repository of suspected ADRs dependent on EudraVigilance (INFARMED, 2018).

The EMA's Pharmacovigilance Risk Assessment Committee (PRAC) is responsible for evaluating and monitoring EudraVigilance safety signals and may recommend regulatory action as a result. It is composed of drug safety experts from MS

regulatory authorities, in addition to scientific experts and representatives of patients and healthcare professionals appointed by the EC (EMA, 2021).

1.3.4. Software tools and Databases

The comprehension and application of PGx tests in clinical practice has been a challenge for many clinicians, but guidelines already exist to assist them in using genetic information for drug selection and dosing.

The Pharmacogenomics Knowledgebase (PharmGKB) is one of the world's leading resources for PGx knowledge and has been adapting and refocusing its mission along with the current revolution in genomic medicine. The PharmGKB website (www.pharmgkb.org/) provides a diverse set of information on PGx, dosing guidelines and drug labels, potentially clinically actionable drug associations, and genotype-phenotype relationships (such as ADR-related genes and variants). It is freely available and accessible to everyone from researchers to clinicians and ordinary citizens (Yoon et al., 2020; Barbarino et al., 2018).

PharmGKB reports on associations to haplotypes, repeats, copy number variations, and insertions and deletions. Variant annotations are manually added by curators for both positive and negative results. Each variant annotation contains a standardized summary sentence describing the results, as well as a free text section and study parameters such as cohort size, association values, and cohort ethnicities. Researchers can use the data for projects such as exploring PGx relationships across the genome, predicting new PGx interactions, or providing a PGx perspective on genotype panels (Barbarino et al., 2018; van der Lee et al., 2020).

As part of its role in the clinical implementation of PGx, PharmGKB has noted guidelines from professional groups, primarily from the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Dutch Pharmacogenetics Working Group (DPWG), which are large consortia. CPIC and DPWG publications represent clinical implementation at the grassroots level and are therefore important to provide. Both primarily focus on pharmacotherapeutic recommendations for a large number of drugs in combination with a patient's predicted genotype or phenotype (Yoon et al., 2020; van der Lee et al., 2020).

CPIC guidelines are available through the CPIC website (https://cpicpgx.org/). They are written in a standardized format, consisting of background information about

the gene, variant, and drug under discussion, information about genetic testing options, and a discussion of the evidence linking genotype to phenotype. Users can enter a genotype or haplotype of interest and receive functional information and dosing recommendations as directed. In this way, the goal is to help physicians understand how available genetic test results should be used to optimize drug therapy (Yoon et al., 2020; Barbarino et al., 2018).

PharmGKB has also developed the Pharmacogenomics Clinical Annotation Tool (PharmCAT) software, with the goal to extract CPIC guideline variants from a genetic dataset, interpret the variant alleles, and generate a report with actionable CPIC dosing recommendations (Barbarino et al., 2018).

The DPWG is an expert group from Netherlands that publishes its pharmacogenetic recommendations based on clinical evidence at www.knmp.nl. Its application is primarily pre-emptive (Lunenburg et al., 2020; Brouwer et al., 2022).

The goal is help physicians to guide therapy based on PGx test result to prevent ADRs. The clinical relevance of the potential adverse drug event, decreased therapeutic response, or other clinical effect resulting from the gene-drug interaction is assessed through scores that are assigned to each combination of predicted genotype or phenotype and a specific drug (Yoon et al., 2020).

1.4. Cytochromes P450 (CYPs)

Cytochromes P450 (CYPs) comprise a superfamily of microsomal heme-thiolate enzymes, which catalyze a high diversity of reactions such as oxidation, peroxidation, and reduction. CYP450 enzymes accept as substrates a wide range of compounds both endogenous (steroids, prostaglandins, and fatty acids) and exogenous (drugs, environmental pollutants, agrochemicals, and other potentially toxic compounds). They play a critical role in some of the most pertinent issues in clinical pharmacology nowadays, as they participate in phase I metabolism of more than 90% of commonly used clinical drugs (biological and vaccines excluded) contributing for inter-individual variability in drug response, drug toxicity and ADRs (Danielson, 2002; Meijerman et al., 2007; Zanger and Schwab, 2013).

CYP450 enzymes are encoded by *CYP* genes, with the wild type allele prevailing in most individuals of the population. Individuals who carry two copies of the wild type allele are *normal metabolizers* (NM). Alternative alleles also exist, and they often

incorporate sequence variants with impact on protein structure or expression leading to altered enzyme activity. Considering specifically CNVs, individuals harboring deleted alleles present decreased or null enzyme activity and are called *intermediate metabolizers* (IM) and *poor metabolizers* (PM) and have a higher risk of drug toxicity. When taking prodrugs, these patients will experience lower availability of the active compound and, therefore, decreased therapeutic effect or treatment failure is expected. On the other hand, individuals with multi-copies of a functional allele show an increased enzyme activity and thus enhanced drug degradation. They are called *rapid or ultra-rapid metabolizers* (UM). *Rapid and ultra-rapid metabolizers* are at risk of ineffectiveness of treatment using standard drug doses because the drug is so rapidly metabolized in the liver, that blood therapeutic concentrations are never achieved or, if a prodrug is administrated, increased toxicity, adverse events and even death can occur (Gaedigk et al., 2017).

The enzymes CYP2C19 and CYP2D6 are important biomarkers once they are the main enzymes involved in the metabolism of most neuropsychotic drugs. Some polymorphisms in these enzymes are responsible for the interindividual variability on drug response.

1.4.1. CYP2C19

The cytochrome P450 enzyme, family 2, subfamily C, polypeptide 19 (CYP2C19) is encoded by *CYP2C19* gene located at 10q23.33 inside the *CYP2C* locus along with *CYP2C8, CYP2C9* and *CYP2C18* genes. All of them have nine exons and share high degree of sequence similarity (Botton et al., 2019).

CYP2C19 is involved in the metabolism of a large number of clinically relevant drugs and drug classes, such as antidepressants, benzodiazepines, mephenytoin, proton pump inhibitors (PPIs), and the antiplatelet prodrug, clopidogrel. Genetic variations in *CYP2C19* (table 1) are responsible for causing deficient drug metabolism and ADRs in patients. Accordingly, this gene is considered by PharmGKB as a Very Important Pharmacogene (VIP) with Tier 1, meaning it is a gene with substantial evidence to support its importance in PGx.

According to CPIC, *CYP2C19*2* and *CYP2C19*3* are the most frequent alleles with decreased activity.

*CYP2C19*2* is the most frequent *CYP2C19* variant in many populations and the second most frequent in Europe. It occurs in exon 5 at position 681 (c.681G>A; rs4244285). This change of a guanine (G) to adenine (A), leads to an aberrant splicing site. The creation of this site shifts the reading frame of mRNA, starting with 215 amino acid residues, and prematurely creates a stop codon for 20 amino acid residues earlier, resulting in a shorter, functionally inactive protein (Scott et al., 2012; Dehbozorgi et al., 2018; Hassani et al., 2018).

The *CYP2C19*3* allele also results in a nonfunctional protein; the variant occurs in exon 4, position 636 and generates a premature stop codon (c.636G>A; rs4986893) (Scott et al., 2012; Dehbozorgi et al., 2018; Hassani et al., 2018).

*CYP2C19*17* is the most frequent allele in European and many other populations. *CYP2C19*17* variant occurs in the promotor region at position -806 with the change of cytosine (C) to thymine (T) (c.-806C>T; rs12248560) leading to the binding of a specific nuclear protein to the 5'-flanking region and consequently in an increased gene transcription and high enzyme activities (Scott et al., 2012; Dehbozorgi et al., 2018; Hassani et al., 2018).

Table 1 – Important *CYP2C19* alleles affecting enzyme function (PharmVar and CPIC).

Allele	Alteration	SNP	Location	Frequency in Europe *	Enzyme function
CYP2C19*2	c.681G>A	rs4244285	Exon 5	14.69%	Loss-of-function
CYP2C19*3	c.636G>A	rs4986893	Exon 4	0.16%	Loss-of-function
CYP2C19*17	c.-806C>T	rs12248560	Promoter	21.54%	Gain-of-function

*Frequency in Europeans reported in CPIC.

CYP2C19 gene also presents CNVs: (i) whole gene deletions affecting the *CYP2C19* gene and two adjacent genes giving rise to non-functional alleles designated *CYP2C19*36* (figure 1) and (ii) partial gene deletions, being described five alleles **37.001*, **37.002*, **37.003*, **37.004* and **37.005* that differ in the number of remaining exons but sharing the common feature of missing exon 1. Since exon 1 encodes amino acids that guide protein to the endoplasmic reticulum, these variants, collectively designated as *CYP2C19*37*, most probably encode non-functional proteins (Botton et al., 2021; Botton et al., 2019).

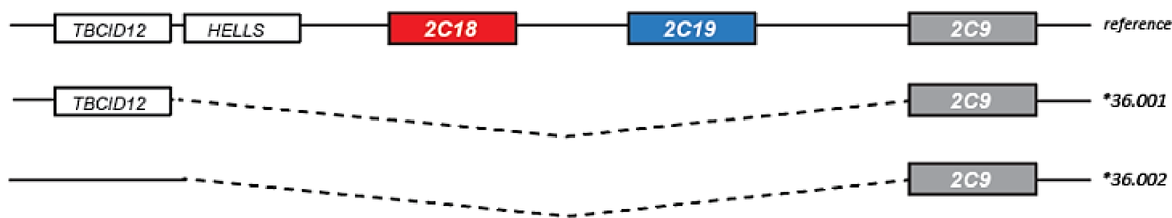


Figure 1 – *CYP2C* locus. Two allelic variants (*CYP2C19**36.001, *CYP2C19**36.002) carrying large deletions involving *CYP2C19* and adjacent genes (Adapted from PharmVar Consortium).

1.4.2. CYP2D6

The cytochrome P450 enzyme, family 2, subfamily D, polypeptide 6 (CYP2D6) is encoded by *CYP2D6* gene located on chromosome 22q13.1. CYP2D6 metabolizes 25% of commonly prescribed drugs, including antidepressants, antipsychotics, and analgesics. According to PharmGKB it is like CYP2C19 a Tier 1 gene (Ramamoorthy, 2010; Ingelman-Sundberg et al., 2007).

This gene is part of the *CYP2D* locus which contains three genes: *CYP2D6*, a functional gene and two pseudogenes *CYP2D7* and *CYP2D8* (Gaedigk et al., 2010) (figure 2).

CYP2D7 and *CYP2D8* are located 9 and 19kb upstream of the *CYP2D6* gene and share 94.2% and 89.1% sequence similarity with *CYP2D6*, respectively (Taylor et al., 2020). These high levels of homology prone to recombination events, so recurrent CNVs in these two genes usually result from abnormal homologous recombination.

One factor that allows *CYP2D6* to be differentiated from the *CYP2D7* pseudogene, is that whereas *CYP2D6* gene is followed by 0.6 kb repeat region and a repetitive sequence referred as REP6, and *CYP2D7* is followed by a 0.6kb repeat, a unique 1.6-kb ‘spacer’ sequence and a repetitive sequence REP7 (figure 2).

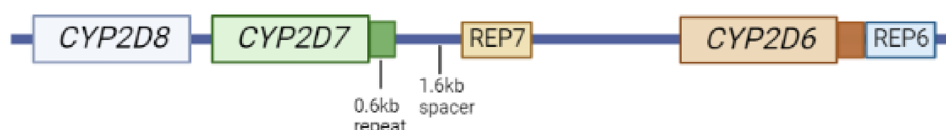


Figure 2 – *CYP2D* gene locus, containing the functional *CYP2D6* gene, and two pseudogenes, *CYP2D7* and *CYP2D8*. There are repeat elements located downstream of *CYP2D6* (REP-6), and *CYP2D7* (REP-7) as well as a *CYP2D7*-derived spacer with 1.6kb.

In addition to the many *SNPs* identified in *CYP2D6* gene, numerous structural variants have also been described in *CYP2D6*, including CNVs (deletions and duplications of genes and structural rearrangements between *CYP2D6* and the pseudogene). Some *CYP2D6* allelic variants also harbor *CYP2D7-derived* sequences known as 'hybrids' and, similarly to *CYP2D7*, all *CYP2D6/CYP2D7* hybrids genes are also followed by a REP7 sequence.

Changes of the number of gene copies can interfere with enzyme activity as an all. Individuals with more than 2 copies of the gene usually have increased amount of enzyme and exhibit higher activity. However, duplications/multiplications (figure 3) do not always result in increased enzyme function, because the phenotype also depends on the functionality of the allele. *CYP2D6*1, CYP2D6*2, CYP2D6*35* are alleles presenting normal function, *CYP2D6*10, CYP2D6*17, CYP2D6*41* reduced function, and *CYP2D6*4*, *CYP2D6*36* are non-functional alleles. Thus, carriers of non-functional allele duplications are *poor metabolizers*, whereas duplications of fully functional genes have been shown to confer *ultrarapid metabolizer* status, except for the tandem allele *CYP2D6*36+*10* (because *CYP2D6*36* is non-functional, and *CYP2D6*10* is a reduced function allele) (Gaedigk et al., 2010). This tandem is typically found in individuals of East Asian ancestry and have the *36 hybrid located upstream of the *10 gene copy.

Duplicate genes have a REP-DUP sequence which is a hybrid containing a 5′ REP6 sequence and a 3′ REP7 sequence, except *CYP2D6*36+*10* that have REP7.

The most common alleles carrying two or more copies of the gene (xN) are *CYP2D6*1xN*, *CYP2D6*2xN* or *CYP2D6*4xN* (Gaedigk et al., 2007).

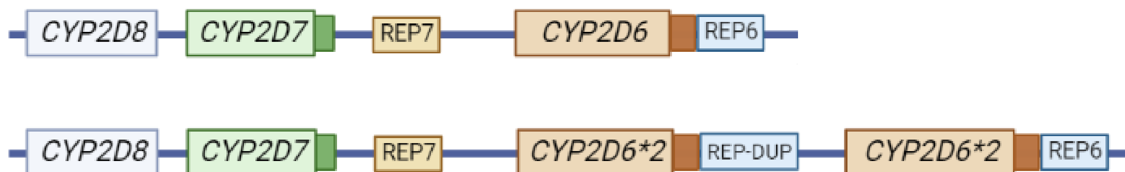


Figure 3 –*CYP2D6* locus and an example of *CYP2D6* gene duplication (*CYP2D6*2x2*). Most common duplication structure entails two (or more) identical genes, and contains the REP-DUP repeat structure, which is *CYP2D6*-like and lacks the spacer.

CYP2D6 also have a full gene deletion allele, defined as *CYP2D6*5*. This allele has breakpoints in the REP7 and REP6 regions, and the region downstream of the *CYP2D7* allele containing the "spacer" is called REPdel (figure 4).

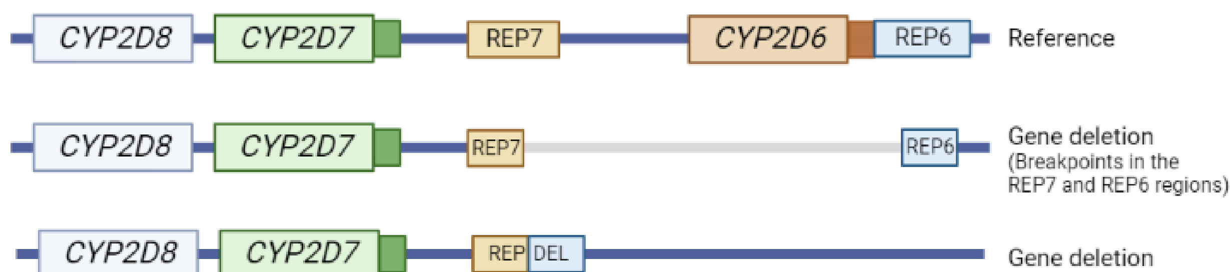


Figure 4 – *CYP2D6* locus and (up) complete *CYP2D6* gene deletion (down). Breakpoints in the REP7 and REP6 regions for generation of the structure REPdel are also represented (middle).

Table 2 presents some of the most frequent *CYP2D6* loss of function and null alleles, in Europe according to CPIC, namely.

Table 2 – Important *CYP2D6* alleles affecting enzyme function (PharmVar and CPIC).

Allele	Alteration	SNP	Location	Frequency in Europe *	Enzyme function
CYP2D6*3	c.775dellA	rs35742686	Exon 5	1.59%	Null function
CYP2D6*4	c. 506-1G>A	rs3892097	Intron 3	18.54%	Null function
CYP2D6*6	c.454delT	rs5030655	Exon 3	1.11%	Null function
CYP2D6*9	c.2615delAAG	rs5030656	Exon 5	2.76%	Loss-of-function
CYP2D6*10	c.100C>T	rs1065852	Exon 1	1.57%	Loss-of-function
CYP2D6*41	c.985+39G>A	rs28371725	Intron 6	9.24%	Loss-of-function

*Frequency in Europeans reported in CPIC.

1.5. Genotyping techniques

The implementation of PGx in the laboratory presents major challenges concerning the selection of molecular biology methods; although many techniques for DNA screening are already available and applied in clinical practice, they are mainly used to identify single nucleotide variants, small deletions, or insertions.

Genotyping in this context is the process of identification of an individual's genotype using a molecular genetics assay, to detect variants that may influence drug metabolism and thus ADRs or lack of efficacy.

Methods used to detect SNVs include Polymerase Chain Reaction (PCR), quantitative real-time polymerase chain reaction (qPCR), RFLP (Restriction Fragment Length Polymorphism), Sanger sequencing, DNA microarrays and more recently Next Generation Sequencing.

To detect CNVs in *CYPs*, the most popular method is the Multiplex Linkage Dependent Probe Amplification (MLPA®), a semi-quantitative, non-automated technique that enables the detection of copy number variations of several DNA sequences, using a single PCR-based multiplex reaction (MRC Holland, 2022).

CNVs can also be detected either by using high-throughput scanning technologies, such as comparative genomic hybridization (CGH) and high-density SNP arrays, or by using relatively low-throughput techniques, such as qPCR (Qin et al., 2008). However, these sequencing methods have limitations and cannot accurately genotype some pharmacogenes, like *CYP2D6*, since these methods use short reads which are not appropriate for genotyping genes with closely related pseudogenes, CNVs and structural rearrangements. The analysis of these type of genes is more complex and reliable testing is difficult (Nofziger and Paulmichl, 2018; Willard, 2015; Beoris et al., 2016).

Long-range PCR (XL-PCR) was found among the most used methods to identified CNVs in *CYP2D6* and it stands as a strong tool to characterize structure variants involving this gene and neighbor pseudogenes. Gaedigk and col. are authors of many papers about this subject as they were pioneers in developing some of the methodologies for characterization of CNVs and deciphering the complexity of the rearrangements *CYP2D6/CYP2D7* (Gaedigk et al., 2010). However, XL-PCR is a method with limitations for routine use, and have a Low/medium success rate, and possibility of incorrect incorporation of dNTPs.

To overcome this barrier, third-generation sequencing approaches have been proposed (Santos et al., 2018; Gulilat et al., 2019; Santos et al., 2022). Nanopore sequencing is a unique and scalable technology that allows direct, real-time analysis of DNA, requiring neither PCR amplification nor nucleotide tagging for detection (Loose et al., 2016).

The advantage of using this technique is that we can sequence the genome integrally, and thus overcome the limitations of other sequencing methods. In addition, since no amplification is required, this method is cheaper, and has a simpler workflow by not having the amplification step and by doing analysis in real time, and it is also possible to sequence simultaneously the two DNA strands making the results more feasible (Loose et al., 2016; Kumar et al., 2019).

But like all methods, this one also has challenges, namely the nucleotides adjacent to the target nucleotide to be sequenced have a role in distorting the electric field, the turnover of the motor enzyme is somewhat random, and the technology is new and still under development (Kumar et al., 2019).

2. OBJECTIVES

Relevant enzymes involved in the metabolism of neuropsychiatric drugs are CYP450 enzymes, namely CYP2D6 and CYP2C19. The genes coding for these enzymes are highly polymorphic and several variants have impact in phenotypic expression. CPIC created PGx clinically actionable guidelines for antidepressants based on *CYP2D6* and *CYP2C19* haplotypes. Studies that correlate particular SNPs and CNVs with different response profiles to drug exposure are welcomed and can be used with marked benefit to personalized medicine, to decrease the incidence of ADRs and drug no-response.

The aim of this study is to genotype a cohort of Portuguese patients taking antidepressant drugs and to predict their impact on therapy.

3. METHODOLOGY

3.1. Subjects

This study enrolled a cohort of 112 patients taking neuropsychiatric medication. These individuals were part of the e_COR project - *Study of the Prevalence of Cardiovascular Risk Factors in the Portuguese Population* (Bourbon et al., 2019).

They were selected from a larger group of individuals representatives of Portuguese Population. The samples were recovered from a repository for studying gene variants related with pharmacokinetics and pharmacogenomics. Patients' data are confidential, and therefore we did not have access to personal data. The local ethics committee of INSA approved the study.

3.2. Sample Preparation

DNA extraction was performed by a salting out method, adapted from the protocol described by Lahiri and Nurnberger, 1991.

The first step was to thaw and homogenize the blood very well and transfer the total volume into a Falcon tube. Then, TKMX, a mixture of TKM1 (10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM MgCl2 and 2 mM EDTA) and TRITON X-100 (Polyethylene Glycol Octyl Phenyl Ether, a non-ionic surfactant or detergent), was added to blood and homogenized vigorously, aiming the red cell lysis. Next, IGEPAL was also added, shaken vigorously, and vortexed. The sample was centrifuged (2200 rpm, 10 min, 18 °C, acceleration-9 and slowdown-7) in order to separate the white blood cells.

The supernatant was discarded and TKM1 was added to wash the pellet, the tube was shaken vigorously to resuspend the pellet, and centrifuged (1600 rpm, 10 min, 18°C, acceleration-9 and slowdown-7). This washing process was repeated two or three times as needed. At this stage, the pellet is washed, containing only leukocytes (WBC) and proteins.

In order to extract DNA, WBCs were lysed by adding TKM2 (10 mM Tris-HC1 pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 0.4 M NaCl and 2 mM EDTA) and SDS 10 % to the pellet which was resuspended and heated for 10 minutes at 55 °C for chemical digestion.

To precipitate the proteins, 5M NaCl was added and then centrifuged (13 200 rpm, 20 min, 18 °C). The supernatant was collected, and for DNA precipitation, absolute

ethanol was added and mixed by gentle shaking with semi-inversion. With a loop, the DNA was rolled, passed in 70 % ethanol to remove excess absolute ethanol, and allowed to dry for about 5 min in the loop. Then, the loop was broken into a tube with TE, where the DNA was stored at 4 °C until further quantification.

DNA quantity and quality was determined by the NanoDrop® spectrometer (ThermoFisher Scientific) using 1 µL of DNA of each sample to evaluate the concentration and purity. The ratio Abs260/Abs280nm, was measured to evaluate the purity of the samples. It should be between 1,8 and 2,0. Values lower than 1,8 indicate a possible contamination with proteins, (absorbing light at 280nm).

Finally, to verify the DNA's integrity, a 1 % agarose on TBE1X, gel electrophoresis (with SYBR Safe dye) was performed for 40 min at 90V.

Three samples of the European Proficiency Control Program were also tested after proper dilution (see annex 1).

3.3. Sample analysis

3.3.1. Multiplex ligation-dependent probe amplification (MLPA)

MLPA is a semi-quantitative method based on hybridization of DNA with specific probes followed by amplification of the hybridized probe and analysis of the resulting PCR products. The objective is to detect copy number changes at the genomic level (gains and losses) compared to control samples and to identify deletions, duplications and amplifications in specific exons within a single run.

A variety of MLPA kits are commercially available, each kit having a variable number of specific probes with different lengths and unique target sequences. In addition to the probes, each kit has two oligonucleotides that hybridize to adjacent sides of the target sequence to increase specificity.

For this study, MLPA was performed using the SALSA MLPA P128-D1 Cytochrome P450 probe mix (#P128-D1, MRC-Holland, Amsterdam) according to the manufacturer's instructions. This commercial MLPA probe mix is an assay for research use only (not validated for diagnostic use) (MRC Holland, 2022), requiring confirmation of results by complementary methodologies.

The P128-D1 probe mix detects deletions or duplications in CYPs (*CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4* and *CYP3A5*) and also in glutathione S-transferases (*GSTM1, GSTP1* and *GSTT1*). In this study we focused only on CYPs, more specifically on *CYP2C19* and *CYP2D6*. The kit contains 52 MLPA probes. Three of them hybridize with sequences of exons 2, 6, and 9 of *CYP2C19* and four for with CYP2D6 sequences of exons 1, 5, 6 and downstream of exon 9.

In addition to the 112 samples, we used 3 controls as reference.

The first step of MLPA is the denaturation reaction. For each of the 112 sample 50 ng of DNA was denatured in a thermocycler at 98 °C for 5 min and then cooled at 25 °C.

The second step of the technique is the hybridization reaction. The mixture of "MLPA Buffer" and "Mix Probe" is prepared according to the number of samples and added to each sample and incubated in the thermocycler at 95 °C for 1min followed by 12-16h of hybridization at 60ºC.

The third step is the ligation reaction which binds the two oligonucleotides together to form the complete probe and ensures that only perfectly paired fragments are amplified in the next step. The reaction mixture was prepared with ligase buffer A, ligase buffer B and ligase-65, and then incubated at 54 ˚C for 15 minutes. The program follows at 98°C for 5 minutes and then pauses at 20 °C.

The last step is the PCR reaction using exon-specific probes with universal tagged primers (table 3). The reaction mixture is prepared with "SALSA PCR primer mix" and added to each tube and amplification is performed in 35 cycles of 30 sec at 95 °C, 30 sec at 60 °C and 60 sec at 72 °C, and then a 20 min final extension at 72 °C and a pause at 15 °C.

The samples were stored protected from light and delivered to the UTI (*Unidade de Tecnologia e Inovação*) with run request. Then, results were analyzed using the software Coffalyser.Net (V.140721.1958).

Table 3 – Probes from the MLPA P128-D1 Cytochrome P450 probe mix kit used to detect CNVs in *CYP2C19* and *CYP2D6* (#P128-C1, MRC-Holland, Amsterdam).

Gene	Partial sequence[a] (24 nt adjacent to ligation site)	Ligation site	Exon
***CYP2C19* (NM_000769.4)**	AGCTCTCAAAAA-TCTATGGCCCTG	203-204 nt	Exon 2
	AAACTTGGTAAT-CACTGCAGCTGA	891-892 nt	Exon 6
	GACACAACTCCT-GTTGTCAATGGA	1438-1439 nt	Exon 9
***CYP2D6* (NM_000106.6)**	AGTGAGGCAGGT-ATGGGGCTAGAA	19-20 nt	Exon 1
	CTGTACCTCCTA-TCCACGTCAGAG	200 nt before exon 5	Exon 5
	CCCATGAACTTT-GCTGGGACACCC	68 nt after exon 6	Exon 6
	CCTGGGCTTCCA-TGGGGCCTTCCC	495 nt after exon 9 reverse	Downstream

Legend: [a]Only partial probe sequences are shown. Complete probe sequences are available at www.mrcholland.com.

3.3.2. Long-range PCR (XL-PCR) for MLPA validation

To validate the deletions and duplications detected in *CYP2D6* by MLPA, XL-PCR was performed as previously described (Gaedgik et al., 2010) using the SequalPrep™ Long PCR kit with dNTPs.

As referred the *CYP2D* locus contains the gene *CYP2D6,* as well as and *CYP2D7* and *CYP2D8* pseudogenes. They share a high degree of sequence similarity. Therefore, it is necessary to have specific primers that discriminate the gene from its pseudogenes.

We used primers from literature to amplify a 6.6kb *CYP2D6*-specific fragment that encompassed the entire gene (fragment A). Further, to ensure that we were amplifying the duplicated genes, we used primers enabling the amplification of the duplicated copy and the REP-DUP sequence together forming a fragment of 8.6kb (fragment D), (figure 5). The primers used were described by Gaedgik et al., 2010.

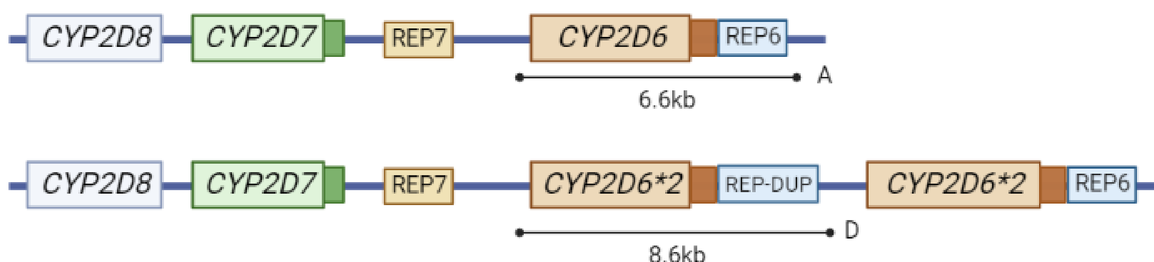


Figure 5 – Representation of *CYP2D6* gene and the amplified fragments (A and D) used to detect gene duplications.

Briefly, we prepared the master mix according to the manufacturer's protocol and primers for fragment A and D (table 4), the mixture was added to each sample and placed in the thermocycler for at 95 ºC for 10 min initially, then 35 cycles (94 ºC for 3 sec, 62 ºC for 30 sec and 72 ºC for 6 min) with a final step at 72 ºC for 20 min and then cooled at 15 ºC.

The PCR products were analyzed by electrophoresis in a 1% agarose gel with SYBR™ Safe DNA Gel Stain (50 min at 90V).

Table 4 – Primers used for XL-PCR.

Fragment	Primers	Product length (kb)
A	F- 5' ATGGCAGCTGCCATACAATCCACCTG 3' R- 5' CGACTGAGCCCTGGGAGGTAGGTAG 3'	6.6
D	F- 5' CCAGAAGGCTTTGCAGGCTTCAG 3' R- 5' CGGCAGTGGTCAGCTAATGAC 3'	8.6

A method for confirmation of the hybrids detected in *CYP2D6* by MLPA, is currently under development.

3.3.3. Sanger sequencing

Sanger sequencing methodology was performed for detection of SNVs, related to decreased or null CYP2D6 or CYP2C19 activities.

First, we identified regions of ***CYP2C19*** gene*,* and polymorphisms, that according to CPIC make up relevant alleles. Thus, three fragments of *CYP2C19* were selected for sequencing in the 112 DNA samples: the 5'UTR region, exon 4 and exon 5 (table 5).

*CYP2C19*2* (located in exon 5) and *CYP2C19*3* (located in exon 4) are the most frequent alleles with decreased activity, and *CYP2C19*17* (located in promoter) is the most frequent allele with an increased activity.

Table 5 – Primers used to amplify *CYP2C19* regions.

Primers	Region to amplify	Product length (bp)
F- 5' GGGGCTGTTTTCCTTAGATAAATAAGT 3' R- 5' AGGACAAAGTCTCCTAATCTTCGA 3'	5'UTR	525
F- 5' CCAGCTAGGCTGTAATTGTTAATTCG 3' R- 5' TGGCAAAGTTCTTTATTTTATGCACAGG 3'	Exon 4	485
F- 5' CCAGAGCTTGGCATATTGTATCTA 3' R- 5' TGTTTAACAGGTCAAGGAGTAATG 3'	Exon 5	352

PCR was performed in a 25 µl reaction volume containing 50-100 ng of genomic DNA, 5x Colorless GoTaq reaction buffer, dNTPs mix (10mM each), $MgCl_2$ solution (25mM), GoTaq DNA Polymerase (2U) and specific primers (10nM) for regions of interest. The following PCR cycling conditions were used: 10 min at 95 °C and 32 cycles at 94 °C for 45 sec, 60 °C for 45 sec and 72 °C for 1 min, followed by 72 °C for 5 min and cooled at 15 °C. Amplification products were electrophoresed on 0,6 % TBE1X agarose gel stained with SYBR™ Safe DNA Gel Stain and visualized on a UV transilluminator.

To sequence ***CYP2D6*** we performed a nested-PCR using the Fragment A as template, in all the samples except those suspected of having hybrids.

The fragments A and D (figure 5) were used as template to perform a nested PCR. Reagents and thermocycler conditions were the same previously used to amplify *CYP2C19*, and specific primers. Fragments of interest were considered exon 1, exons 3+4, exon 5 and exon 6 (table 6), because according to CPIC, these are the regions with the most frequent variants with impact for *CYP2D6* alleles definition.

Table 6 – Primers used to amplify *CYP2D6* regions.

Primers	Region	Product length (bp)
F- 5' TGTAAAACGACGGCCAGTGCTGGTGTGCTGAGAGTG 3' R- 5' CAGGAAACAGCTATGACCACTGCCAAGTCCAGCTCCAC 3'	5'UTR Exon 1	507
F- 5' TGTAAAACGACGGCCAGTCATAGGGTTGGAGTGGGTG 3' R- 5' CAGGAAACAGCTATGACCATGTCCTTTCCCAAACCCATC 3'	Exon 3-4	591
F- 5' TGTAAAACGACGGCCAGTTGAGGTCAGTGGTAAGGACAGG 3' R- 5' CAGGAAACAGCTATGACCCCAATTCTGCACCTGTCAGC 3'	Exon 5	431
F- 5' TGTAAAACGACGGCCAGTGTTGGACCAGTGCATCACC 3' R- 5' CAGGAAACAGCTATGACCCTGGTCAAGCCTGTGCTTG 3'	Exon 6	359

PCR products from *CYP2C19* regions and nested PCR products from *CYP2D6* were purified with ExoProStar incubating for 15 min at 37 °C and 15 min at 80 °C and cooled at 15 °C.

Sequencing of the purified products, using forward and reverse primers, was conducted with the BigDye® Terminator v1.1 Cycle Sequencing kit according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA, USA). The amplification consisted of pre-denaturation at 96 °C for 1 min, followed by 30 cycles of denaturing at 96 °C for 10 sec, annealing at 58 °C for 5 sec, and extension at 60 °C for 4 min, and cooled at 15 °C. DNA sequencing was performed in ABI PRISM® 310 Genetic Analyzer by capillary gel electrophoresis with fluorescence detection. Sequence electropherograms were aligned and analyzed using the software Pregap4® and Gap4®.

4. RESULTS

Several methods were sequentially used to genotype the cohort of 112 individuals taking neuropsychiatric drugs. Figure 6 displays the flowchart of this study.

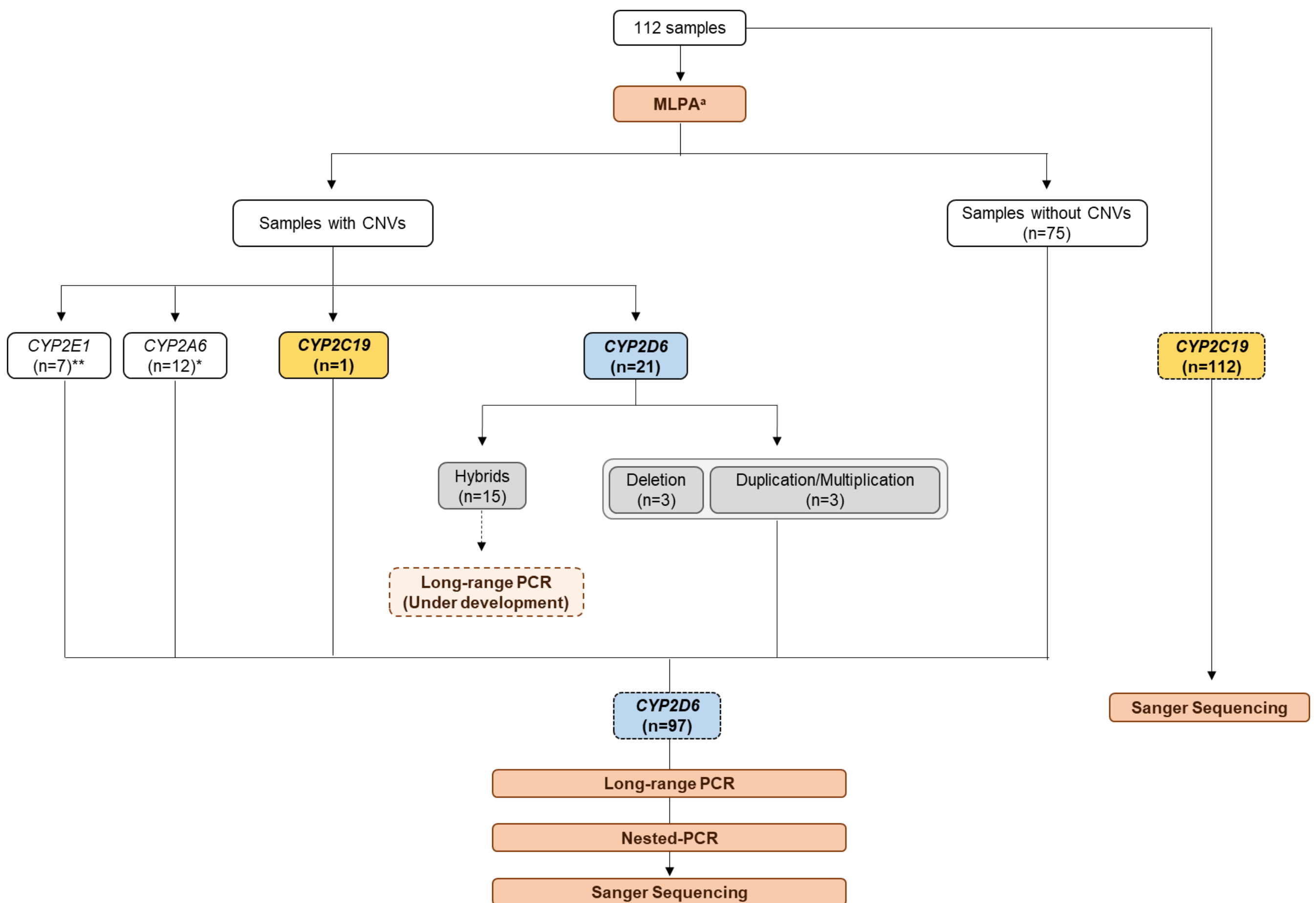


Figure 6 – Flowchart of the study.

Legend: [a]MLPA P128 kit has probes that hybridize with CYPs and also with glutathione S-transferase (GST) genes. For this study the focus was only on CYP genes; *2 sample had also alterations in *CYP2D6* and 1 sample in *CYP2E1;* **1 sample had also alteration in *CYP2D6.*

4.1. MLPA

MLPA analysis covered 11 genes belonging to the cytochrome P450 superfamily, (*CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4* and *CYP3A5*).

Seventy-five samples fully hybridized with all the probes of the kit, as did the controls. The remaining 37/112 presented abnormal results on four genes, *CYP2E1, CYP2A6, CYP2C19*, and *CYP2D6*, namely:

- duplication of the signal with all the probes for ***CYP2E1*** (7 samples). One of these samples also showed alterations in *CYP2A6* and another in *CYP2D6* assay.

- 12 samples had alterations of ***CYP2A6***: (i) two samples had half of the signal with all probes compatible with complete gene deletion of one allele, (ii) seven did not hybridize with the probe targeting exon 1, suggestive of a recombinant event (iii) two samples presented duplication of the signal with all the probes suggestive of a gene duplication event and (iv) one sample showed duplication in one probe only (targeting exon 1) which points to a recombinant event.

- 1 sample presented half of the signal in one probe (targeting exon 2), compatible with partial gene deletion of one ***CYP2C19*** allele.

- 21 samples showed results suggesting of copy number changes in ***CYP2D6***: (i) three had half of the signal with all probes compatible with complete one allele *CYP2D6* deletion, (ii) two samples presented duplication of the signal of all the probes suggesting a duplication of the *CYP2D6* gene, (iii) one exhibited a very high signal (4 copies) for all probes compatible with a gene multiplication event, (iv) fifteen samples were suspected of recombinant events because they have duplication in some probes (13 samples presented a signal of 3 copies in exon 1 and one sample had a signal of 4 copies also in exon 1, and one sample presented a signal of 3 copies in three probes, that target namely exon 1, exon 5, and exon 6).

Using bioinformatic tools and the dedicated databases from CPIC and PharmVar it was possible to characterize some of the above alleles. The heterozygous deletion involving exon 2, found in *CYP2C19* is compatible with the *CYP2C19*37* allele (Botton et al., 2019). It is expected that with this rare allele would have the same effect on drug response as the well-known no function alleles defined by sequence variants (e.g., *CYP2C19*2, *3).*

With *CYP2D6* results, we can observe a allelic frequency of 1.34% with a deletion allele, 0.89% with a duplication allele, 0.45% with multiplication allele and 6.70% with supposed *CYP2D6/CYP2D7* hybrids alleles.

The characterization of the alleles involved in *CYP2D6* deletion and duplication, found by MLPA, was done by Sanger sequencing (see below).

CNVs involving *CYP2A6* and *CYP2E1* can be considered incidental findings as we identified them as lateral results of the work done. Although this study does not focus on these *CYPs*, based on bioinformatics tools it is also possible to classify the alleles. CNVs of *CYP2A6* should correspond to *CYP2A6*4* (gene deletion) and duplications *CYP2A6*1xN,* samples suspected of having hybrids need further analysis to determine the alleles. The *CYP2E1* CNVs are likely to be *CYP2E1*1xN* duplications.

Full MLPA results are presented in table A2 in annex 2.

4.2. CYP2C19

Sanger sequencing of the 3 selected fragments of *CYP2C19,* on the 112 samples, enabled to identify the most common single base variants in this gene. It was performed to look for the most frequent SNPs of *CYP2C19* (table 7).

The most frequent allele in the studied population was *CYP2C19*17* [1] (harboring the c.-806C>T variant), with an allelic frequency of 35.27%. Out of the 112 samples, 5 were heterozygous (C/T), 37 homozygous with this SNP (T/T) and 70 WT homozygous (C/C).

The individuals that have a diplotype with an increase function allele and a normal function allele (*1/*17), are *rapid metabolizers*; the 37 patients with two increase function alleles (*17/*17) corresponding to *ultrarapid metabolizer* phenotype, and the patients with normal alleles have a *normal metabolizer* phenotype.

The frequency of *CYP2C19*2* allele (harboring the SNP rs4244285, c.681G>A) was 12.05%. Twenty-five patients were heterozygous for this SNP (G/A), 1 homozygous (A/A) and 86 were homozygous WT (G/G).

[1] *CYP2C19**17 can be mixed with *CYP2C19**4 because *4 can present a C or a T at position c.-806. However *CYP2C19**4 was not considered in this study because its frequency in Caucasians is around 1/500 whereas *CYP2C19**17 is 2,1/10 in Caucasians.

Individuals carrying a no function allele and one normal allele (*1/*2) have an *intermediate metabolizer* phenotype, those who have two no function alleles (*2/*2) are *poor metabolizers*, while he individuals with two normal alleles are *normal metabolizers*.

No patients were found harboring the SNV (c.636G>A, rs 4986893).

Six of the 112 patients carried both alleles, *CYP2C19*17* and *CYP2C19*2,* (*2/*17). It was not possible to detect if the variants are in *cis* or *trans.*

In general, 4.46% (n=5) of the studied population have an *ultrarapid metabolizer* phenotype, 27.68% (n=31) are *rapid metabolizers*, 44.64% (n=50) are *normal metabolizers*, 22.32% (n=25) are *intermediate metabolizers* and 0.89% (n=1) are *poor metabolizers* (table 7).

The sample that gave a partial deletion in MLPA, showed no SNPs in the sequencing fragments. So, this patient is likely an *intermediate metabolizer* carrying a no functional allele and one normal allele (*1/*37).

Table 7 – Attribution of likely phenotypes for antidepressants based on diplotypes (CPIC guidelines).

Gene	Allele/diplotype	No. of patients (n=112)	Percentage of patients (%)	Likely phenotype
CYP2C19	*17/*17	5	4.5	Ultrarapid metabolizer
	*1/*17	31	27.7	Rapid metabolizer
	*1/*1	50	44.6	Normal metabolizer
	*1/*2, *2/*17[a], *1/*37	25	22.3	Intermediate metabolizer
	*2/*2	1	0.9	Poor metabolizer

Legend: [a] The predicted metabolizer phenotype for the *CYP2C19**2/*17 genotype is a provisional classification. The currently available evidence indicates that the *CYP2C19**17 increased function allele is unable to completely compensate for the *CYP2C19**2 no function allele.

4.3. CYP2D6

To confirm the MLPA results and further characterize *CYP2D6* alleles, XL-PCR was performed. Gel electrophoresis enabled the visualization of amplified fragments: A is a 6.6kb long fragment that encompassed the entire *CYP2D6* gene; fragment D is 8.6kb XL-PCR product that includes the *CYP2D6* duplicated copy and the REP-DUP sequence (Figure 5).

Figure 7 shows an agarose gel with 3 samples. There was amplification of the fragment A (6.6 Kb), in the samples 1 and 3. Sample 3 is a no CNVs case and sample 1 according to MLPA harbors a full gene deletion in heterozygote state. It should be highlighted that without MLPA sample 1 could be wrongly interpreted as no CNVs. Sample 2 amplified both fragments A (6.6 kb) and D (8.6 kb) confirming the presence of normal allele and a duplicated one as found in MLPA assay.

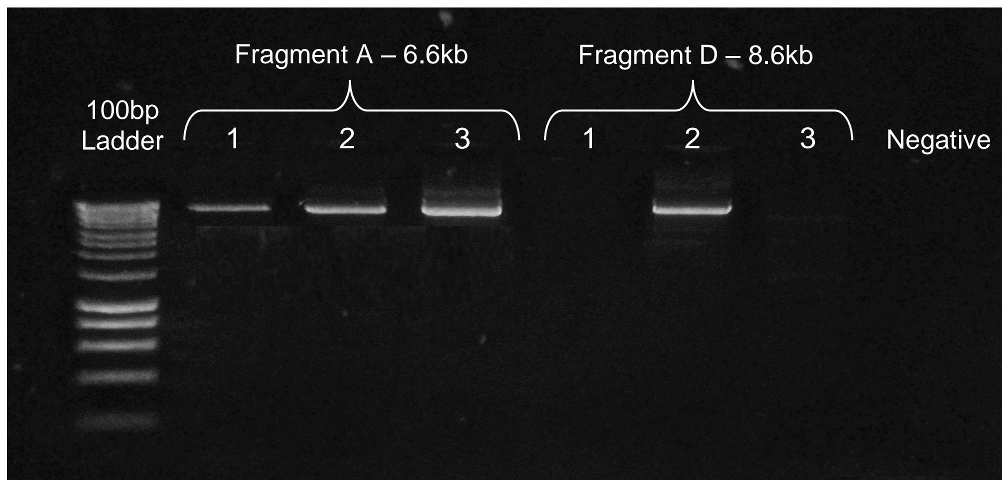


Figure 7 – Agarose gel to check XL-PCR amplification and evaluate the presence of *CYP2D6* duplications and deletions.

Legend: Sample 1 – *CYP2D6* (heterozygous) deletion; Sample 2 – *CYP2D6* gene duplication; Sample 3 – No CNVs.

From the initial 112 samples, the 15 suspected of hybrid *CYP2D6/CYP2D7* recombination were not sequenced. From the remaining 97, in 19 it was not possible to amplify the DNA by XL-PCR (the reaction was prepared three times, with no result). Thus, we only got results from 78 samples.

The 78 samples were sequenced and genotyped according to the SNVs found, as we did for *CYP2C19*. We used CPIC guidelines, PharmVar, and the website https://databases.lovd.nl/shared/genes.

Seven distinct *CYP2D6* alleles were identified based on the SNVs (c.100C>T; c.696T>C; c.180+34G>C; c.180+41C>A; c.180+43C>G; c.180+47T>C; c.180+53A>C; c.180+65A>G; c.180+52G>C; c.180+130G>T; c.408G>C; c.886C>T; c.181-40T>G; c.1174-9C>T; c.1457G>C; c.181-41T>G; c.271C>A; c.281A>G; c.294C>G; c.1392C>T; c.31G>A; c.82C>T; c.506-1G>A; c.985+39G>A; c.841delAAG) (Table 8).

The allele frequency of *CYP2D6*4* (harboring the variant c.506-1G>A) was 8.04%. Sixteen samples were heterozygous (G/A), for this SNP (rs3892097) whereas one was homozygous (A/A) for this alteration and 61 were WT homozygous (G/G).

*CYP2D6*41* revealed an allelic frequency of 4.46%. No homozygous were found for the c.985+39G>A variant, rs28371725. Heterozygous (G/A) were 10, and 68 WT homozygous (G/G).

*CYP2D6*9* revealed an allelic frequency of 0.45%. No homozygous were found for the deletion c.2615delAAG, rs5030656. One heterozygous case was identified and 77 WT homozygous.

The normal allele *CYP2D6*2* revealed an allelic frequency of 15.63%. Three homozygous, 29 heterozygous cases were identified and 80 WT homozygous.

The allelic frequency of WT allele (*CYP2D6*1)* was not calculated because most of the cases it is valuated based on an exclusion process.

In one case we cannot figure out which allele of the *4/*9 genotype has duplicated. Further, the diplotype *2/*44 is very rare, so we didn't find the corresponding phenotype. Nevertheless, as allele *2 is a normal function, and *44 is no function the expected phenotype associated to this diplotype could be *intermediate metabolizer* as it is *1/*5.

In global, 55.36% (n=65) of the studied population are *normal metabolizers*, 12.50% (n=14) are *intermediate metabolizers*, 0.89% (n=1) are *ultrarapid metabolizer* and 0.89% (n=1) are *poor metabolizers.* For the remaining 30.36% (n=34) of the study population, we couldn't determine the phenotype.

Genotyping *CYP2D6* gene was much more complicated than *CYP2C19*, because it is very polymorphic. Many alleles had the same variants and sometimes is difficult to characterize the genotype. For example, some cases with 4 variants where 3 of them are heterozygous and 1 is homozygous, can originate 4 different chromosomal combinations (figure 8).

Table 8 – Results of *CYP2D6* genotyping and phenotype of 78 patients taking neuropsychiatric drugs.

Diplotype	Number of patients (n=112)	Frequency	Phenotype
***1/*1**	15	13.4%	NM
***1/*2**	15	13.4%	NM
***1/*4**	11	9.8%	NM
***1/*41**	6	5.4%	NM
***1/*35**	4	3.6%	NM
***2/*35**	3	2.7%	NM
***2/*2**	3	2.7%	NM
***2/*41**	3	2.7%	NM
***4/*35**	3	2.7%	IM
***2/*4**	3	2.7%	IM
***2/*5**	2	1.8%	IM
***1/*36**	2	1.8%	IM
***4/*5**	1	0.9%	PM
***2/*36**	1	0.9%	IM
***2/*44**	1	0.9%	IM
***35/*35**	1	0.9%	NM
***35/*41**	1	0.9%	NM
***1/*2xN**	1	0.9%	UM
***1/*4xN**	1	0.9%	IM
***4/*9 (xN)**	1	0.9%	IM
Hybrids and samples without results	34	30.4%	?

Legend: UM, Ultrarapid metabolizer; IM, Intermediate Metabolizer; NM, Normal Metabolizer; PM, Poor Metabolizer.

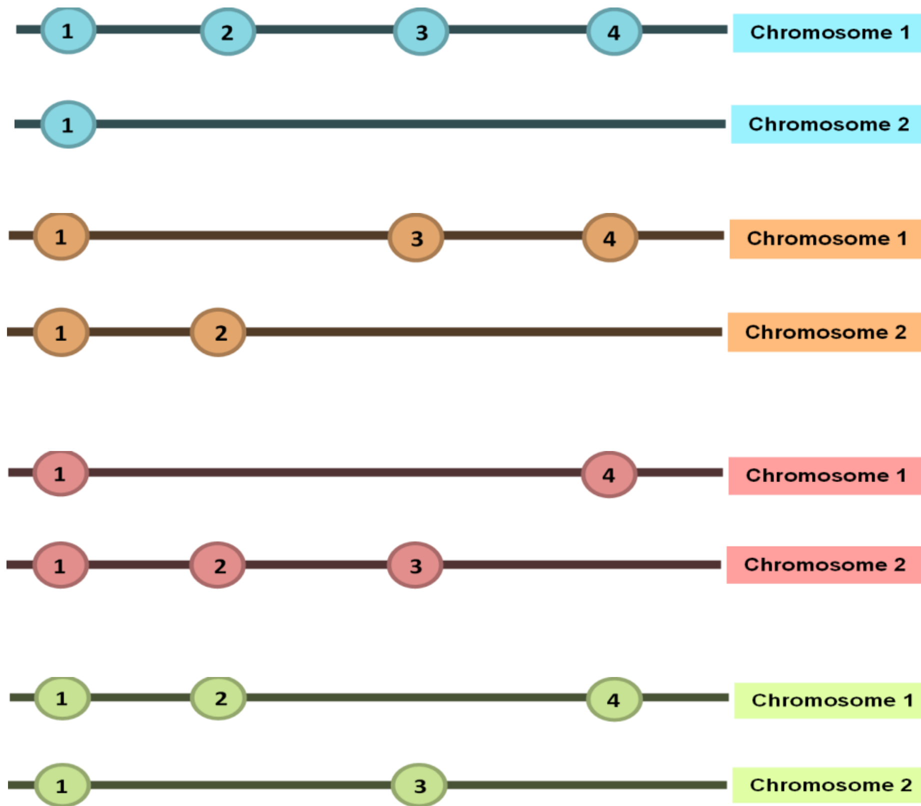


Figure 8 – Combinations of four SNPs, 3 in heterozygosity and 1 in homozygosity.

XL-PCR was also performed to sort the hybrids with specific primers; however it involves optimization of several XL-PCR reactions and without having positive controls to validate the results it was not possible to have final results yet.

5. DISCUSSION

5.1. Selection of *CYP2C19* and *CYP2D6* genes

Our study group takes neuropsychiatric medication. This includes antidepressant and antipsychotic clinical drugs. Thus, it was necessary to select from the published guidelines and literature the genes involved in the metabolism of these drugs relevant for a pharmacogenetic approach.

According to FDA, polymorphisms located in *CYP2C19* and *CYP2D6* genes are included as pharmacogenetic biomarkers in antidepressant drug labeling (Center for Drug Evaluation and Research, 2022; Corponi et al., 2018), due to the possibly higher risk of ADRs, toxicity, drug interactions, and the possible necessity of dose adjustments in intermediate metabolizer and replacement in poor metabolizers. So, clinical guidelines with recommendations based on *CYP2C19* and *CYP2D6* functional groups are provided by experts from CPIC and DPWG.

CPIC has developed evidence-based clinical guidelines for SRRIs and tricyclic antidepressants, recommending adjusted dosing based on *CYP2D6* and *CYP2C19* metabolic status (Hicks et al., 2015; Hicks et al., 2017). There are currently no CPIC guidelines for antipsychotics, but the Dutch Pharmacogenetics Working Group provides guidelines for aripiprazole, haloperidol, pimozide and zuclopenthixol based on CYP450 genotype. It is important to use information provided by these databases to prescribe the dose that fits the individual's metabolism, especially for *poor metabolizers* and *ultrarapid metabolizers*, in order to avoid ADRs and treatment poor response. The phenotype of the individual can be evaluated by pharmacokinetics assays or inferred from the knowledge of his/her genotype.

Literature supports that CYP2D6 and CYP2C19 are the two main enzymes involved in the metabolism of antidepressant and antipsychotic drugs (Corponi et al., 2018; Zanger and Schwab, 2013).

5.2. Interest of this study

It is often difficult to have the right drug, for the right person at the right dose, at the right moment (personalized pharmacotherapy), often leading to inappropriate choices.

In this context, PGx can help because it allows to know for each individual whether that drug will be metabolized as expected in clinical trials, faster or slower, and therefore, with the help of guidelines, establish a personalized therapy plan for greater therapeutic efficacy and fewer ADRs. In addition, the use of biomarkers for specific therapies is seen as a path to a more efficient and cost-effective healthcare system.

Reduction of ADRs in Portuguese patients with psychiatric disorders can be achieved by implementing pharmacogenetic testing for *CYP2C19* and *CYP2D6* during drug selection and dosing. This requires as a start point to know which are the most common variants in these genes in the Portuguese population well as to establish appropriate validated methodologies.

5.3. Analytic Strategy

Since we currently do not have a method available in the laboratory that can detect SNVs and CNVs, a flowchart was established to guide the various tests in a sequential manner in order to get the most out of the information.

With this study, in addition to Sanger sequencing to detect SNVs, we also performed MLPA which allowed to detect deletions and duplications. Sanger sequencing cannot detect CNVs, so if we didn't use MLPA, we wouldn't have detected the *CYP2C19* partial deletion and the *CYP2D6* duplications, deletions, and hybrids.

MLPA distinguishes itself from other methodologies because it allows for the detection of rearrangements, which most other methodologies cannot (Sahajpal et al., 2021; Norris et al., 2016). However, it has disadvantages that include difficulty in accurately characterizing some alleles as we don’t know the beginning and end of a deletion or duplications). This kit for *CYPs* is not validated for diagnosis, and therefore changes must be confirmed by another independent technique for clinical use, such as XL-PCR. It cannot detect changes outside the target sequence of the probes, such as inversions or copy number neutral translocations. Furthermore, as well as most commercially available genotyping tests, does not detect rare or de novo variants There are also external constraints, namely when we do not have positive control samples for validation and because international PGx control programs are still very recent and do not yet include rarer variants and hybrids.

Sequencing the samples with duplications in *CYP2D6,* we characterized the alleles based on SNPs, where we obtained the genotypes *1/*2xN and *1/*4xN. However, for the suspected hybrid samples it was not possible to characterize the alleles because they were too complex. Hybrids are derived from recombination, such as gene conversion, which is a mechanism involving a unidirectional transfer of genetic sequence information from a donor sequence to a highly homologous acceptor sequence, or by crossover which is the exchange of genetic material between the non-interfering chromosomes of homologous chromosomes, occurring predominantly during meiosis (Samanthi, 2021).

Due to methodology constraints, some questions remain to be answered, namely although we know that deletion of the full *CYP2D6* gene has breakpoints in the CYP2D7-REP7 and CYP2D6-REP6 regions, it still remains unknown if the three deletion alleles cases identified have identical breakpoints, and the hybrids detailed sequence.

This study allowed a better understanding of the advantages and limitations of the methodologies used and the difficulty of pharmacogenetic testing

5.4. Allelic frequencies

In the present study we evaluated genetic alterations in *CYP2C19* and *CYP2D6* in 112 patients. We compared the allele frequencies found in this study with the reported CPIC frequency in distinct ethnic groups, and with allele frequencies in Portugal obtained in other studies (Figure 9).

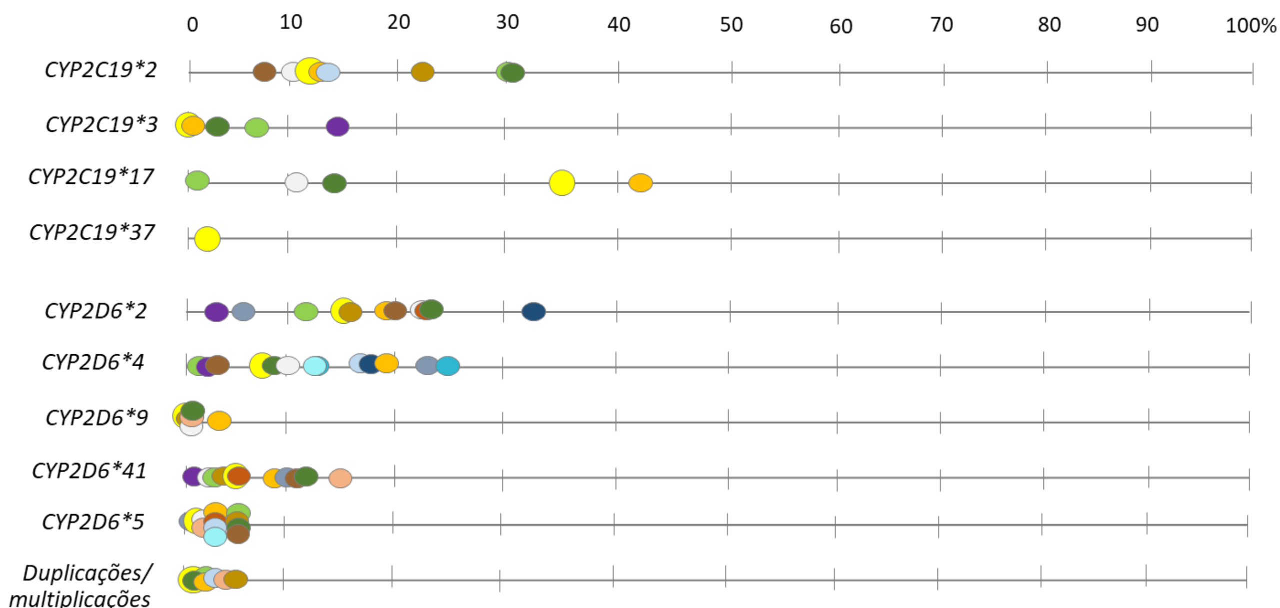
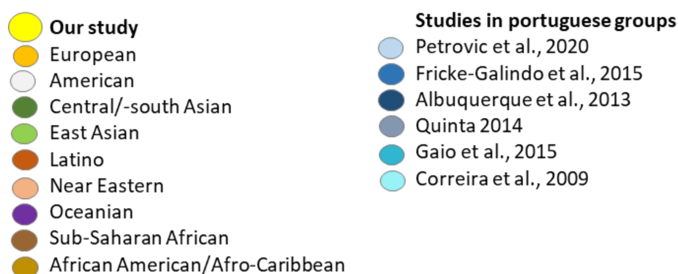


Figure 9 – Allele frequencies obtained in this study and other ethnic groups according to CPIC as well as in previous studies performed in the Portuguese population.

Legend:



In this study, 12.05% of the studied population carried the *CYP2C19*2* allele, frequency similar to Native Americans (10.84%), American Caucasians (13.01%) and Central Europeans (13.07%), and higher than North Africans (8.65%). However lower than African Americans (22.31%), East Asians (30.26%) and South Asians (30.34%) (Fricke-Galindo et al., 2015). Petrović et al., 2020, performed a systematic analysis of the frequency distribution of clinically relevant *CYP2D6* alleles in Europe, and obtained an allele frequency of 13.4% for *CYP2C19*2* in Portugal.

Concerning the *CYP2C19*17*, 35.27% of the individuals carried this allele, translating a frequency closed to Mediterranean-South Europeans (42.00%) but higher than Native Americans (10.95%), South Asians (13.74%) and East Asians (0.96%) (Fricke-Galindo et al., 2015).

*CYP2C19**3 is an allele with 0.16% of frequency in European population according CPIC guidelines, and we didn't find this allele in the 112 Portuguese individuals tested. Australian Caucasians and Scandinavians had reported a frequency of 0% for this allele, in contrast, the allele is more common in Native Oceanians (14.42%), East Asians (6.89%) and North Asians (4.43%) (Fricke-Galindo et al., 2015).

In the studied population, the distribution of *CYP2C19* phenotype was the following: 44.64% of the patients were *normal metabolizers*, 27.68% were *rapid metabolizers*, 22.33% were *intermediate metabolizers*, 4.46% were *ultrarapid metabolizer*, and 0.89% were *poor metabolizers*.

The accurate phenotype prediction for *CYP2D6* was more difficult due to gene complexity; effectively the gene has a high number of known allelic variations, including CNVs and structural rearrangements involving *CYP2D6* and *CYP2D7* genes.

The most frequent alleles in this study were the normal function alleles *CYP2D6*1* and *CYP2D6*2* with normal function, and the non-functional *CYP2D6*4.*

15.63% of the studied population carried the *CYP2D6*2* allele. Together with *CYP2D6*1* (the reference haplotype for *CYP2D6*) they represent the haplotypes known as *normal metabolizers*. When compared with previous Portuguese studies the frequency found in the current investigation is lower than that obtained by Albuquerque et al., 2013 (33.33%), and higher than 6.4% (data obtained by Quinta 2014). Albuquerque studied 300 unrelated healthy Caucasian adult volunteers using a real-time PCR approach and confirmed the results by long PCR and PCR-RFLP, Quinta et al. also performed Real-time PCR in 55 Portuguese Caucasian adult patients. Our results are much more comparable with *CYP2D6*2* allele European frequencies (19%).

According with CPIC data, our frequency for the *CYP2D6*2* allele was similar to African American (16%) and East Asian populations (12%) and higher than allele frequency of Oceanian (4%). In contrast, it was lower than Sub-Saharan African (20%), American population (22%), Latino population (23%), and Central/South Asian (29%).

8.04% of the individuals carried the *CYP2D6*4*, a non-functional allele that contributes to the majority of *poor metabolizers* in Caucasian population. According with

CPIC this allele shows a frequency similar to Central/South Asian (9%) and American population (10%). East Asian (1%), Oceanian population (2%) and Sub-Saharan African (3%) have lower frequencies. In European population *CYP2D6*4* exhibits an higher frequency than that found in the present study (19%).

*CYP2D6*4* frequency found in this study (8,0%) is lower, than the reported in other studies performed in Portuguese population. The systematic study of Petrović et al., 2020, proposed a *CYP2D6*4* allele frequency of 17% in Portugal. Quinta 2014 published a frequency of 23.6% and Gaio et al., 2015 (that performed a RFLP-PCR), 24.6% in Centre of Portugal, 12.9% in the South Portuguese region, and 13.3% at Lisbon and Tagus Valley Further. Albuquerque et al., 2013 and Correia et al., 2009 reported higher frequencies, 18.2% and 13.3% respectively.

*CYP2D6*41* revealed a minimum allelic frequency of 4.46%. This frequency is similar to African American (4%) and Latino populations (5%), reported on CPIC guidelines and higher than Oceanian (1%), American (2%) and East Asian (2%). In contrast it is lower than Near Eastern (15%), Central/South Asian (12%), Sub-Saharan African (11%) and European populations (9%). Quinta, 2014 reported *CYP2D6*41* allele frequency of 10% in the Portuguese population.

*CYP2D6*9* revealed an allelic frequency of 0.45%. Similar to African American (0.44%), American (0.44%), Central/South Asian (0.30%), Near Eastern (0.38%) populations but lower than European population (2.76%) This allele was not found yet in Oceanian and Sub-Saharan African populations. This is the first study reporting *CYP2D6*9* frequency on Portuguese population.

A frequency of 1.34% was found for the allele *CYP2D6*5* (full gene deletion), which is similar in American (2%), Near Eastern (2%), European (3%) and Latino populations (3%). It is lower than the 5% reported in African American, Central/South Asian, East Asian and Sub-Saharan African populations.

Previous studies on Portuguese population revealed comparable results: Quinta 2014 0.9%; Petrović et al., 2020 2.6% and Correia et al., 2009, 2.8%. Correia studied 100 Portuguese unrelated healthy Caucasian adults using a long PCR approach.

Duplications and multiplications have a combined frequency of 1.34%. According with CPIC guidelines the allele frequency in European population is 1.93%, 1.54%, in East Asian populations and 1.13%in Central/South Asian. African American (5.17%), and Near Eastern populations (4.30%), have frequencies a little higher when compared to

our study. Duplications and multiplications in *CYP2D6* were not found yet in Oceanian population. Petrović et al., 2020, reported a frequency of *CYP2D6* duplication in Portugal of 3%.

We identified 6.70% of supposed hybrid *CYP2D6/CYP2D7* alleles. Since there is several different *CYP2D6* hybrids, it is difficult compare frequencies.

Allelic frequencies differ by ethnic group. However, there are also differences in the frequencies reported on several studies done in Portugal, which can be explained by the fact that we still have 19 samples with no results. If we had the results of these samples the allele frequencies might have been different. So (i) our frequencies are the minimum frequencies of each allele presented; (ii) the percentages in some comparative studies may not have considered the CNVs and differentiate *CYP2D6* pseudogenes; (iii) Portugal has a wide range of individuals from other ethnic backgrounds, and this also interferes in the allele frequencies from group to group studied.

Of the 112 patients, 62 are *normal metabolizers*, 14 *intermediate metabolizers*, one is *ultrarapid metabolizer* and one *poor metabolizer*. The remaining 34 patients of the study population, we couldn't infer a phenotype.

5.5. Clinical implications

Clinical trials have been performed relating *CYP2D6* and *CYP2C19* phenotypes with adverse reactions. Many of the studies have shown that *poor metabolizers* of *CYP2D6* or *CYP2C19* have higher serum levels of antidepressants and antipsychotics, compared to *normal metabolizers*, thus having substantially increased exposure to the drug and higher risk that a toxic event occur and ADRs, while *ultrarapid metabolizer* present more often lower concentration levels of the drug, due to faster metabolism leading to insufficient response to treatment (Huezo-Diaz et al., 2012; Chen et al., 2015; Olson et al., 2017; Milosavljevic et al., 2021).

Using CPIC guidelines for SSRIs and TCAs, we can access the recommended adjusted dose based on *CYP2D6* and *CYP2C19* metabolic status.

SSRIs are primary treatment options for major depressive and anxiety disorders. SSRIs that are known *CYP2C19* substrates are: citalopram, escitalopram, and sertraline, and the known substrates for *CYP2D6* are: fluoxetine, fluvoxamine, paroxetine, and sertraline. The more common ADRs by SSRIs include central nervous system effects

(e.g., insomnia, headache), gastrointestinal dysfunction, and sexual dysfunction; however, the incidence of side effect occurrence differs with each drug.

For ultrarapid metabolizers of *CYP2D6*, is recommended select alternative drug not predominantly metabolized by *CYP2D6*. The same for ultrarapid metabolizers of *CYP2C19*: the prescription of an alternative drug not predominantly metabolized by *CYP2C19* is recommended. For poor metabolizers is recommended to consider an alternative drug not predominantly metabolized by the same gene, or a 50% reduction of standard starting dose.

TCA *CYP2C19* substrates are amitriptyline, clomipramine, doxepin, imipramine and trimipramine, and TCA substrates for *CYP2D6* include amitriptyline, clomipramine, duloxetine, and doxepin. Common adverse effects to TCA treatment include anticholinergic, central nervous system, and cardiac effects.

For poor and ultrarapid metabolizers of *CYP2D6*, itis recommend avoiding tricyclic drugs use due to potential lack of efficacy and consider alternative drug not metabolized by *CYP2D6*, and for intermediate metabolizers is advised to consider a 25% reduction of recommended starting dose.

Concerning *CYP2C19*, rapid, ultrarapid and poor metabolizers are avoided to used tertiary amine due to potential for sub-optimal response and recommended an alternative drug not metabolized by *CYP2C19*.

The combination of *CYP2D6* and *CYP2C19* phenotypes will influence the use of certain drugs, and it is necessary to use guidelines according to the drug.

In addition to dosage based on genotype, there is also influence from drug interactions. Patients treated for psychiatric disorders often require multiple medications, which can influence tricyclic plasma concentrations, side effects, and treatment failure.

Patients using multiple drugs, influence tricyclic plasma concentrations, side effects, and therapeutic failure. A recent data show that up to 20% of patients treated for depression can convert from normal metabolizer status to poor CYP2D6 metabolizer, by taking strong CYP2D6 inhibitors, and thus they should be treated similarly to poor *CYP2D6* metabolizers. For example, patients taking amitriptyline in combination with a potent *CYP2D6* inhibitor, such as fluoxetine, may have dramatic increase in amitriptyline plasma concentrations (Preskorn et al., 2013).

In addition, patients with increasing age, liver disease, and reduced renal function may require reduced doses of TCAs.

Not forgetting *CYP2C19* also metabolize other drugs besides antidepressants, like the antifungal voriconazole, the proton pump inhibitors (PPIs), anticonvulsants like diazepam and phenytoin and the antimalarial agent proguanil and therefore *CYP2C19* genotype will also influence their metabolism. The same happens with *CYP2D6* that participates in the metabolism of more than 20% of common clinical other drugs like the antipsychotic risperidone, several antiarrhythmics, beta-blockers like carvedilol and metoprolol, opioids namely codeine and tramadol, the anticancer drug tamoxifen, and other and other xenobiotics.

5.6. Future perspectives

To overcome the difficulties in methodologies for PGx, the third-generation sequencing methods can be the future for high-throughput sequencing, by enabling native single molecule sequencing, avoiding typical amplification PCR-based bias as well as detecting DNA methylation associated to highly repetitive genomic regions.

Nanopore sequencing has the ability to produce substantially longer reads in one only run, when compared to the second generation, allowing detection of repetitive regions, larger deletions, translocations and inversion events (Liau et al., 2019; Ammar et al., 2015). So, the use of this method would allow us to clarify and confirm the more difficult haplotypes and also to characterize hybrids.

While still in active development, third-generation sequencing has shown promising results for high-accuracy, content-rich whole-genome sequencing and can therefore be a viable solution for the evolution of pharmacogenomics at the structural variants level (Goenka et al., 2022). Although there is still few data using long-reading sequencing to detect CNVs in CYPs (Liau et al., 2019, van der Lee et al., 2022, Charnaud et al., 2022), it has successfully finding structural variants in other applications (Halliwell et al., 2021; Nowak et al., 2021; Xia et al 2021; Magi et al., 2019; Aganezov et al., 2020; Spealman et al., 2020; Magini et al., 2022).

6. CONCLUSION

This study enabled to evaluate the frequency of *CYP2C19* and *CYP2D6* polymorphisms in users of psychotropic medication, including tricyclic antidepressants and antipsychotics, with implications for a better adequacy of the dose-response relationship to the medication, ensuring greater safety and efficacy regarding the use of medications, improving the quality of life of patients.

Poor metabolizers or *ultrarapid metabolizer* phenotypes are the most critical phenotypes for drug metabolism as they cause adverse drug reactions and/or drug unresponsiveness. Some *intermediate metabolizers* also require attention for adaptative dosing. Therefore, due to inter-individual differences it is useful to genotype patients and then use guidelines according to genotype to determine the most effective drug and dose, avoiding ADRs, time to therapy and clinical costs.

With a more detailed characterization of the Portuguese population, the goal is to improve drug safety, dosing recommendations, and pharmacovigilance, since environmental and ethnic factors vary between locations.

However, there is an urgency to implement novel technologies and approaches, that improve characterization of complex genetic alterations, such *CYP2D6* hybrids, and a better correlation of them with phenotype and consequently if they cause ADRs. Nanopore sequencing can be a solution for these difficulties, in one single run read one or more genes of interesse without break DNA chain.

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ANNEXES

ANNEX 1

Three genomic DNA samples were sent for laboratory validation. This is an external quality assessment (EQA) for Pharmacogenetics and tests a laboratory's ability to detect clinically significant variants in the context of molecular pharmacology.

It is designed for panel testing, although participating laboratories may still use specific tests for variants defined in small numbers, or single genes.

This company sends together a panel of genes with important variants that will influence the metabolism of the drug. Personal information is also provided, as well as the patient's medical condition and therapy used.

Figure A1 is a schematic diagram of the samples study process.

First, we confirmed the identification of the samples and read the information on the document that they carry. Next, we did the quantification to then prepare the dilutions getting concentrations between 50ng/uL-100ng/uL.

Based on the patients' therapies, we selected the genes that are involved in the metabolisms of the drugs used, and thus tested them. Table A1 shows the variants that were sought for the selected genes in each sample.

For all genes we performed the PCR method followed by Sanger sequencing to check for the existence of the SNPs.

To check for the c.6986A>G variant in *CYP3A5*, it was necessary to implement a new methodology in the laboratory to detect SNPs in this, the RFLP-PCR method, since the SNP to be analyzed was in an intronic zone. Below is more detail about the method performed.

To detect deletion and duplication/multiplication in *CYP2D6*, the MLPA method was performed.

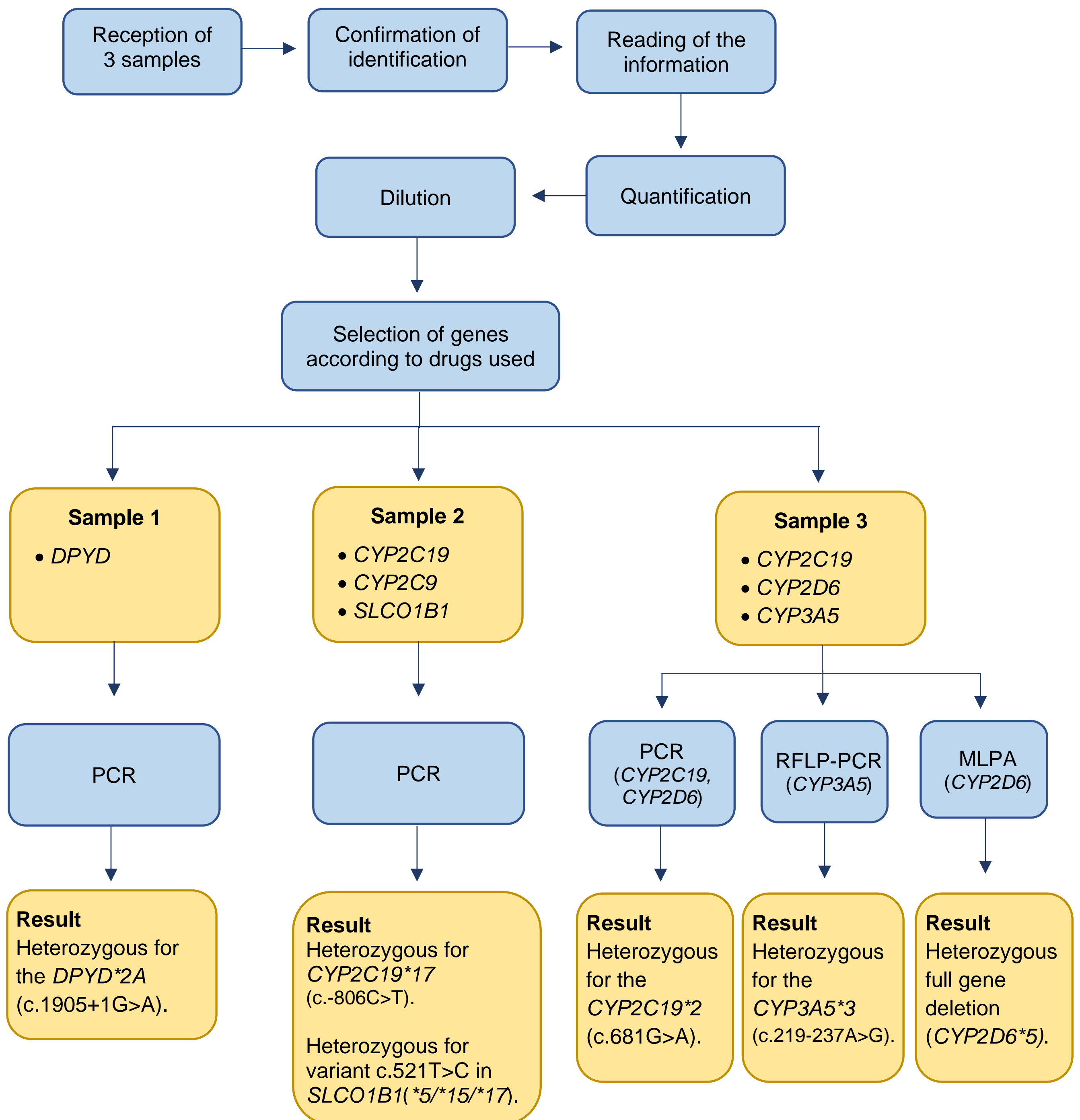

Figure A 1 - Schematic diagram of the samples study process.

Table A 1– Panel of variants to analyze in the selected genes.

Sample	Gene	Genomic position	Allele	RS ID	Wild Type	Result

1	DPYD	NM_000110.4(DPYD):c.1905+1G>A	*2A	rs3918290	G	Heterozygosity
		NM_000110.4(DPYD):c.1679T>G	*13	rs55886062	T	Normal
		NM_000110.4(DPYD):c.2846A>T	X	rs67376798	A	Normal
		NM_000110.4(DPYD):c.1236G>A	X	rs56038477	G	Normal
2	CYP2C19	NM_000769.4(CYP2C19):c.681G>A	*2	rs4244285	G	Normal
		NM_000769.4(CYP2C19):c.636G>A	*3	rs4986893	G	Normal
		NM_000769.4(CYP2C19):c.1A>G	*4A/B	rs28399504	A	Normal
		NM_000769.4(CYP2C19):c.1297C>T	*5	rs56337013	C	Normal
		NM_000769.4(CYP2C19):c.395G>A	*6	rs72552267	G	Normal
		NM_000769.4(CYP2C19):c.358T>C	*8	rs41291556	T	Normal
		NM_000769.4(CYP2C19):c.431G>A	*9	rs17884712	G	Normal
		NM_000769.4(CYP2C19):c.680C>T	*10	rs6413438	C	Normal
		NM_000769.4(CYP2C19):c.-806C>T	*17	rs12248560	C	Heterozygosity
	SLCO1B1	NM_006446.5(SLCO1B1):c.521T>C	*5/*15/*17	rs4149056	T	Heterozygosity
	CYP2C9	NM_000771.4(CYP2C9):c.430C>T	*2	rs1799853	C	Normal
		NM_000771.4(CYP2C9):c.1075A>C	*3	rs1057910	A	Normal
		NM_000771.4(CYP2C9):c.1080C>G	*5	rs28371686	C	Normal
		NM_000771.4(CYP2C9):c.1003C>T	*11	rs28371685	C	Normal
3	CYP2C19	NM_000769.4(CYP2C19):c.681G>A	*2	rs4244285	G	Heterozygosity
		NM_000769.4(CYP2C19):c.636G>A	*3	rs4986893	G	Normal
		NM_000769.4(CYP2C19):c.1A>G	*4A/B	rs28399504	A	Normal
		NM_000769.4(CYP2C19):c.1297C>T	*5	rs56337013	C	Normal
		NM_000769.4(CYP2C19):c.395G>A	*6	rs72552267	G	Normal
		NM_000769.4(CYP2C19):c.358T>C	*8	rs41291556	T	Normal
		NM_000769.4(CYP2C19):c.431G>A	*9	rs17884712	G	Normal
		NM_000769.4(CYP2C19):c.680C>T	*10	rs6413438	C	Normal
		NM_000769.4(CYP2C19):c.-806C>T	*17	rs12248560	C	Normal
	CYP2D6	NM_000106.6(CYP2D6):Dup/multiplication	*xN	-	-	Normal
		NM_000106.6(CYP2D6):c.2549delA	*3	rs35742686	-	Normal
		NM_000106.6(CYP2D6):c.1846G>A	*4	rs3892097	G	Normal
		NM_000106.6(CYP2D6):Gene deletion	*5	-	-	Heterozygosity
		NM_000106.6(CYP2D6):c.1707delT	*6	rs5030655	-	Normal
		NM_000106.6(CYP2D6):c.1758G>T	*8	rs5030865	G	Normal
		NM_000106.6(CYP2D6):c.2615delAAG	*9	rs5030656	-	Normal
		NM_000106.6(CYP2D6):c.100C>T	*10	rs1065852	C	Normal
		NM_000106.6(CYP2D6):c.1758G>A	*14A/B	rs5030865	G	Normal
		NM_000106.6(CYP2D6):c.1023C>T	*17	rs28371706	C	Normal
		NM_000106.6(CYP2D6):c.2988G>A	*41	rs28371725	G	Normal
	CYP3A5	NM_000777.5(CYP3A5):c.6986A>G	*3	rs776746	A	Heterozygosity
		NM_000777.5(CYP3A5):c.14690G>A	*6	rs10264272	G	Normal
		NM_000777.5(CYP3A5):c.27131_27132insT	*7	rs41303343	-	Normal

RFLP-PCR

We followed the protocol described by Fukuen et al., 2002.

PCR was performed with the initial denaturation program at 95ºC for 10min, 37 cycles (30s at 94ºC, 30s at 56ºC and 30s at 72ºC) final extension at 72ºC for 5min and cooling down to 15ºC, we obtain the following PCR product:

PCR product:
CTTTAAAGAGCTCTTTTGTCTCTCAATATCTCTTCCCTGTTTGGACCACATTACCCTTCATCATATG
AAGCCTTGGGTGGCTCCTGTGTGAGACTCTTGCTGTGTGTCACACCCTAATGAACTAGAACCTAAGG
TTGCTGTGTGTCGTACAACTAGGGGTATGGATTACATAACATAATGATCAAAGTCTGGCTTCCTGG
(200bp)

We digest the PCR product with the enzyme DdeI.

DdeI restriction enzyme site:

5´. . . C▼T N A G . . . 3´
3´. . . G A N T▲C . . . 5´

Below, the restriction enzyme cut sites are presented in the sequence in yellow, and in red is marked the SNP (c.6986A>G) and in black the primers. We can see that there is already a natural enzyme cut site in the sequence, if there is the SNP there will be 2 cut sites.

CTTTAAAGAGCTCTTTTGTCTC|TCAATATCTCTTCCCTGTTTGGACCACATTACCCTTCATCATAT
GAAGCCTTGGGTGGCTCCTGTGTGAGACTCTTGCTGTGTGTCACACCCTAATGAACTAGAAC**C|TAA**
GGTTGCTGTGTGTCGTACAACTAGGGGTATGGATTACATAACATAATG**ATCAAAGTCTGGCTTCCTG**
G

If *CYP3A5*1*, there is two fragments:

CTTTAAAGAGCTCTTTTGTCTCTCAATATCTCTTCCCTGTTTGGACCACATTACCCTTCATCATATGAAG
CCTTGGGTGGCTCCTGTGTGAGACTCTTGCTGTGTGTCACACCCTAATGAACTAGAACC (129bp)
+
TAAGGTTGCTGTGTGTCGTACAACTAGGGGTATGGATTACATAACATAATGATCAAAGTCTGGCTTCCTG
G (71bp)

If *CYP3A5*3*, there is three fragments:

CTTTAAAGAGCTCTTTTGTCTC (22bp)
+
TCA**G**TATCTCTTCCCTGTTTGGACCACATTACCCTTCATCATATGAAGCCTTGGGTGGCTCCTGTGTGAG
ACTCTTGCTGTGTGTCACACCCTAATGAACTAGAACC (107bp)
+
TAAGGTTGCTGTGTGTCGTACAACTAGGGGTATGGATTACATAACATAATGATCAAAGTCTGGCTTCCTG
G (71bp)

Result:

With sample 3 we ran the gel samples that we already knew the genotype to serve as

controls. The result for sample 3 was heterozygous for *CYP3A5**3, having the diplotype *1/*3. This individual is predicted to be an intermediate metabolizer according to Clinical Pharmacogenetics Implementation Consortium (CPIC).

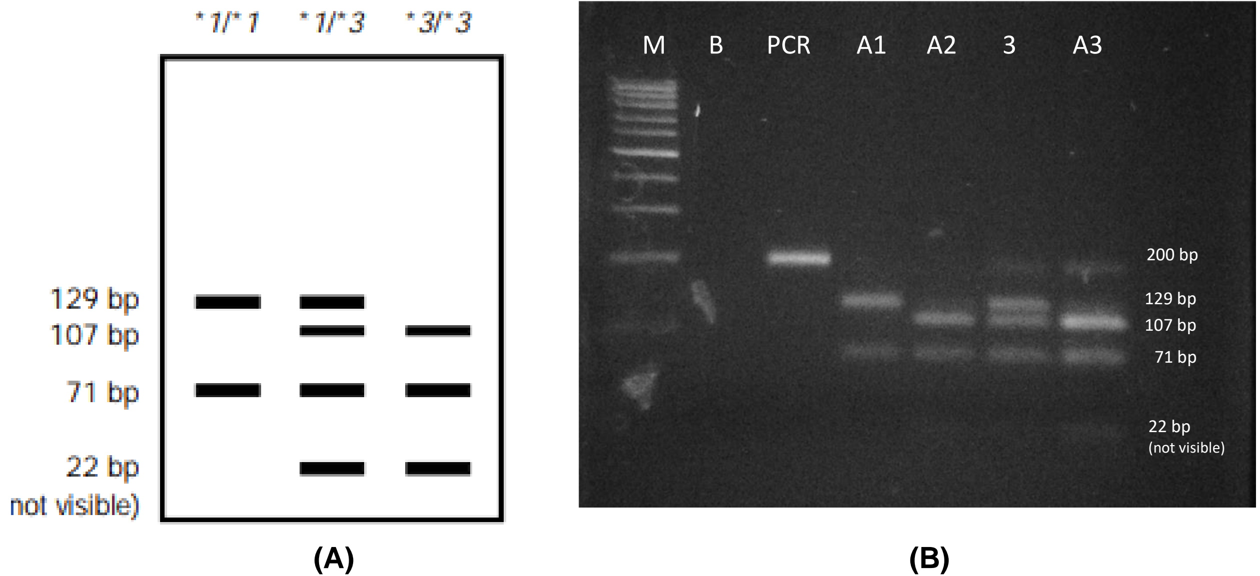

Figure A 2 – (A) Figure adapted from Fukuen et al., 2002. **(B)** Electrophoresis gel performed for sample 3 to detect the existence of the *CYP3A5**3 allele. Legend: PCR, PCR product. A1, A2 and A3 are samples with the result already known, serving as controls (A1-*1/*1, A2-*3/*3, A3-*3/*3). 3, sample 3. B, blank. M, marker.

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ANNEX 2

Table A 2 - Results of the MLPA CYPs probes in the 112 patients.

	CYP2C19-2	CYP2C19-6	CYP2C19-9	CYP2E1-5	CYP2E1-6	CYP2E1-8	CYP1A1-3	CYP1A1-2	CYP1A1-1	CYP2A6-5	CYP2A6-3	CYP2A6-2	CYP2A6-1	CYP2D6-down	CYP2D6-6	CYP2D6-5	CYP2D6-1
A1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C
A2	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A3	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A4	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A5	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/1	1/1	1/1	3 C
A6	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A7	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A8	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A9	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A10	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A11	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A12	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A13	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A14	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	4 C	4 C	4 C	4 C
A15	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A16	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A17	1/0	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A18	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A19	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A20	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A21	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/0	1/0	1/0
A22	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A23	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A24	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A25	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/0	1/0	1/0	1/1	1/1	1/1	1/1
A26	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A27	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/0	1/0	1/0	1/1	1/1	1/1	1/1

A28	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A29	1/1	1/1	1/1	3 C	3 C	3 C	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A30	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C	3 C	3 C	3 C	1/1	1/1	1/1	1/1
A31	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C	1/1	1/1	1/1
A32	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C
A33	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A34	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A35	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A36	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/1	1/1	1/1	1/1
A37	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C	3 C	3 C	3 C	1/1	1/1	1/1	3 C
A38	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C	1/1	1/1	1/1	1/1
A39	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A40	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A41	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A42	1/1	1/1	1/1	3 C	3 C	3 C	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A43	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A44	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A45	1/1	1/1	1/1	3 C	3 C	3 C	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A46	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A47	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A48	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A49	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A50	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A51	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A52	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A53	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A54	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A55	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A56	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A57	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A58	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A59	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A60	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C
A61	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A62	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A63	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A64	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A65	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/0	1/0	1/0
A66	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1

A67	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/0	1/0	1/0
A68	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A69	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A70	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A71	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/1	1/1	1/1	1/1
A72	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A73	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A74	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A75	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A76	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/1	1/1	1/1	1/1
A77	1/1	1/1	1/1	3 C	3 C	3 C	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/1	1/1	1/1	1/1
A78	1/1	1/1	1/1	1/1	1/1	3 C	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A79	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A80	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A81	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/1	1/1	1/1	1/1
A82	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A83	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A84	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C
A85	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C
A86	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C
A87	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A88	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A89	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C
A90	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C	3 C	3 C
A91	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C	3 C	3 C	3 C
A92	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A93	1/1	1/1	1/1	3 C	3 C	3 C	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C
A94	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A95	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A96	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A97	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A98	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	4 C
A99	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A100	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A101	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C	3 C	3 C	3 C
A102	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A103	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A104	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C
A105	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1

A106	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A107	1/1	1/1	1/1	3 C	3 C	3 C	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A108	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/0	1/1	1/1	1/1	1/1
A109	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A110	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
A111	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	3 C
A112	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1

Legend: C, Copies.