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PRECISION MEDICINE IN PSYCHIATRY: PHARMACOGENETICS OF ANTIDEPRESSANTS

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Abstract

La depressione maggiore (DM) è una patologia altamente invalidante che colpisce circa 300 milioni di persone nel mondo. Nonostante siano state sviluppate numerose terapie farmacologiche per il trattamento della DM, ad oggi la percentuale di successo dei farmaci antidepressivi è piuttosto bassa. Infatti, solo il 30% dei pazienti risponde adeguatamente al primo trattamento antidepressivo, mentre una percentuale simile non risponde neppure dopo numerosi interventi farmacologici e sviluppa una depressione resistente ai trattamenti (TRD).

Negli ultimi anni, numerosi studi hanno esaminato il ruolo della genetica nella risposta agli antidepressivi e le potenzialità della possibile implementazione della farmacogenetica (PGx) nella pratica clinica, al fine di ottimizzare la risposta ai trattamenti farmacologici, sia in termini di sicurezza che di efficacia terapeutica. Tuttavia, i risultati ad oggi disponibili sono ancora contrastanti e ulteriori studi sono necessari per comprendere meglio il possibile impatto della PGx nel trattamento della DM.

Lo scopo primario di questa tesi – che comprende 5 studi principali – è quello di approfondire il ruolo della PGx nel trattamento della DM, attraverso un approccio sia clinico che molecolare, e comprendere meglio il contributo delle varianti geniche nei meccanismi causativi della TRD.

Nel primo studio è stato descritto lo sviluppo di un test di PGx che fornisce informazioni potenzialmente utili al fine di indirizzare verso il miglior trattamento antidepressivo per ogni paziente, sulla base del background genetico individuale. Infatti, combinando linee guida e specifiche informazioni di PGx (sia di farmacocinetica che farmacodinamica) presenti in letteratura, è stato sviluppato un algoritmo che, in base al profilo genetico del paziente, classifica gli antidepressivi prescrivibili in tre categorie: “da utilizzare come prima scelta”, “da utilizzare con attenzione” e “da utilizzare con estrema attenzione”. L’utilità clinica del test nel predire correttamente la risposta agli antidepressivi è stata successivamente valutata in un secondo studio qui descritto, che ha evidenziato la capacità dell’algoritmo nell’identificare correttamente i trattamenti antidepressivi che hanno maggiore probabilità di fallire in uno specifico paziente.

Nel terzo e nel quarto studio sono state effettuate valutazioni preliminari sul ruolo di geni candidati, noti per il loro coinvolgimento nella PGx degli antidepressivi, nello sviluppo della TRD. In particolare, il terzo studio si è focalizzato su geni con un ruolo ben noto nella PGx degli antidepressivi, ovvero alcuni membri della famiglia dei citocromi P450 e il trasportatore della serotonina *SLC6A4*. Questa

analisi ha rivelato una maggiore probabilità di sviluppare resistenza agli antidepressivi tra gli individui caratterizzati da un metabolismo "ultrarapido" del citocromo *CYP2C19*. Sempre al fine di caratterizzare i meccanismi genetici alla base della TRD, il quarto studio si è focalizzato su ulteriori varianti geniche precedentemente associate alla risposta agli antidepressivi presenti nel database PharmGKB, rivelando un'associazione significativa tra la TRD e quattro polimorfismi a singolo nucleotide localizzati nei geni *HTR2A*, *GNB3*, *PAPLN*.

L'ultimo studio riportato in questa tesi aveva come scopo la valutazione di possibili alterazioni della lunghezza dei telomeri dei leucociti nei pazienti con TRD (affetti da DM o da disturbo bipolare). I risultati ottenuti hanno rivelato un accorciamento dei telomeri in presenza di TRD, in confronto ad individui di controllo, non affetti da patologie psichiatriche.

In conclusione, i risultati ottenuti da questo lavoro supportano l'implementazione della PGx nel trattamento della DM ed evidenziano la necessità di ulteriori studi per comprendere i meccanismi alla base dello sviluppo della TRD, in un'ottica di un'applicazione della medicina di precisione in psichiatria.

Major Depressive Disorder (MDD) is a severe and debilitating disease affecting approximately 300 million people worldwide. Although several efforts have been made to develop pharmacological therapies for MDD, the success rate of treatments performed with currently available antidepressants (ADs) is low. Specifically, adequate response to the first AD treatment is observed in approximately 30% of patients, and a similar proportion do not achieve remission even after several pharmacological interventions and are defined as affected by Treatment Resistant Depression (TRD).

In the last years, novel pharmacogenetic (PGx) approaches have been developed with, at least in part, contrasting results concerning their efficacy, highlighting the need for further investigations to fully elucidate their contribution in driving treatment choice in MDD.

In this context, the main purpose of this work – comprised of 5 main analyses – is to provide additional insights into the potential role of PGx in MDD treatment, using both clinical and molecular approaches, and to elucidate the genetics underpinnings of TRD.

The first analysis focused on the development of a non-commercial PGx test able to address clinical decision-making toward the best AD treatment for each patient depending on specific genetic information. Relying on customized PGx guidelines and taking into account the patient's genetic background (pharmacokinetics- and pharmacodynamics-related genes), our PGx algorithm classified the most widely used ADs in Italy in three categories: "use as first choice", "use with caution", and "use with extreme caution". The clinical usefulness of our PGx algorithm in predicting response to AD treatment has been evaluated in the second analysis described here, which confirmed the ability of the PGx test in correctly identifying ADs that have a higher likelihood of treatment failure in a specific patient.

The third and fourth analyses aimed to carry out a preliminary evaluation regarding the role of pre-selected candidate genes, previously associated with AD response, in TRD. In particular, the former focused on variants with a known role in PGx of ADs, such as members of the cytochrome P450 family and the serotonin transporter coding gene *SLC6A4*, revealing a significant association between the *CYP2C19* ultrarapid metabolizer phenotype and TRD. The latter of these studies followed a similar approach and focused on genetic variants with a low level of evidence of association with AD response in the PharmGKB database, revealing a significant association between TRD and four single nucleotide polymorphisms in *HTR2A*, *GNB3*, and *PAPLN*.

The fifth analysis reported here aimed at assessing leukocyte telomere length (LTL) in treatment-resistant patients diagnosed with MDD or bipolar disorder. Results revealed a generally shorter LTL in these patients compared with non-psychiatric controls.

In conclusion, results obtained in this PhD thesis work further confirm the usefulness of PGx in MDD treatment and highlight the need for additional efforts aimed at a better understanding of the genetic background underlying TRD and at translating research findings into treatment optimization and drug resistance prevention strategies in the clinical practice.

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1. List of abbreviations

AD: antidepressant
AIC: Akaike information criterion
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AES: antidepressant efficacy survey
AESES: antidepressant efficacy and side effect survey
AS: activity score
BD: bipolar disorder
BDI: Beck's depression inventory
BMI: body mass index
CI = confidence interval
CNV: copy number variations
CO-MED trial: combining medications to enhance depression outcome trial
CPIC: clinical pharmacogenetics implementation consortium
CT: cycle threshold
CYP: cytochrome P450
DDNOS: depressive disorder not otherwise specified
DPWG: Dutch pharmacogenetics working group
DSM-V: diagnostic and statistical manual of mental disorder, fifth edition
FDA: food and drug administration
FIBSERS: frequency, intensity, and burden of side effects rating scale
gDNA: genomic DNA
GAPP-MDD: genomic applications partnership program-major depressive disorder
GENDEP project: genome-based therapeutic drugs for depression project
GUIDED trial: genomic used to guide depression decisions trial
GWAS: genome wide association study
HAMD-17: 17-item Hamilton depression rating scale
IM: intermediate metabolizer
IMPACT trial: individualized medicine: pharmacogenetics assessment and clinical treatment trial
IQR: interquartile range
ISPC: international SSRI pharmacogenomics consortium
LTL: leukocyte telomere length
MDD: major depressive disorder
MADRS: Montgomery-Asberg depression rating scale
MAOIs: monoamine oxidase inhibitors
MARS study: Munich antidepressant response signature study
NASSAs: noradrenergic and selective serotonergic antidepressants
NDRIs: noradrenaline and dopamine reuptake inhibitors
NM: normal metabolizer
NMDA: n-methyl-d-aspartate
NRIs: noradrenaline reuptake inhibitors

PCR: polymerase chain reaction
PGRN-AMPS: pharmacogenomic research network antidepressant medication pharmacogenomic study
PGx: pharmacogenetics/pharmacogenomics
PharmGKB: pharmacogenomics knowledge base
PHQ-9: patient health questionnaire 9
PM: poor metabolizer
PRS: polygenic risk score
QIDS-C16: 16-item quick inventory of depression symptomatology
QoL: quality of life
RCT: randomized controlled trial
RM: rapid metabolizer
rTMS: repetitive transcranial magnetic stimulation
SARIs: serotonin antagonist and reuptake inhibitors
SCID-I: structured clinical interview for DSM-IV axis-I Disorder
SNP: single nucleotide polymorphism
SNRIs: selective noradrenaline and serotonin reuptake inhibitors
SSRIs: selective serotonin reuptake inhibitors
STAR*D trial: sequenced treatment alternatives to relieve depression trial
TAU: treated as usual
TCAs: tricyclic antidepressants
TESI: treatment emergent suicidal ideation
TGTG: treated with genetic test guide
TRD: treatment resistant depression
UM: ultrarapid metabolizer
VNTR: variable number of tandem repeats
WES: whole exome sequencing
WGS: whole genome sequencing
5-HT: 5-hydroxytryptamine (serotonin)
5-HTT: 5-hydroxytryptamine transporter
5-HTTLPR: serotonin-transporter-linked polymorphic region

2. Introduction

2.1. Major Depressive Disorder

2.1.1. An overview on Major Depressive Disorder

Major depressive disorder (MDD) is the most common mental disease and one of the leading causes of disability worldwide. Beside the huge impact on patients' lives, MDD influences individuals social and working functioning, and is one of the main contributors to the overall global disease burden (Mental and Collaborators 2022).

In accordance with the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), MDD is defined as a mental state of persistence for a minimum of two weeks of depressed mood, loss of interest or pleasure, characterized by at least four among several additional symptoms, such as changes in weight, appetite, energy or sleep, psychomotor alterations, sense of guilt, difficulty in concentrating or in making decision, or suicidal ideations or attempts (Otte et al. 2016).

The first-line medical approach to manage MDD is based on the administration of antidepressants (ADs), which include several different molecules that exert their therapeutic effect by restoring one or more biological mechanisms altered in MDD. Because of their high efficacy and low risk to induce adverse side effects, the selective serotonin reuptake inhibitors (SSRIs) are considered the first-choice ADs against MDD. Briefly, SSRIs act inhibiting the serotonin (5-HT) transporter and consequently increasing the levels of 5-HT in the synaptic cleft, thus promoting the stimulation of post-synaptic neurons. Other ADs, as the tricyclic antidepressants (TCAs) and the inhibitors of monoamine oxidase (MAOIs) exert their effects mainly by modulating the monoaminergic system. Depending on whether their structure include a secondary or tertiary amine, TCAs increase serotonin or noradrenaline levels through inhibition of their reuptake. Also, they can modulate other mechanisms involved in MDD, such as the expression of neurotrophic factors, synaptic plasticity, glutamatergic neurotransmission, and inflammatory system. On the contrary, MAOIs increase monoamine levels in the brain and enhance the stimulation of postsynaptic receptors by inhibiting monoamine oxidases, which are involved in degradation of norepinephrine, serotonin, and dopamine. Although their efficacy as ADs has been widely proved, the use of TCAs and MAOIs is strongly limited because of their high probability of side effects (Maffioletti et al. 2020).

Other commonly used ADs include selective noradrenaline and serotonin reuptake inhibitors (SNRIs), noradrenaline (NRIs) and noradrenaline/dopamine reuptake inhibitors (NDRIs), serotonin

antagonist and reuptake inhibitors (SARIs), and noradrenergic and selective serotonergic antidepressants (NaSSAs) (Maffioletti et al. 2020). Given the role of glutamatergic system in MDD pathophysiology, also N-methyl-D-aspartate (NMDA) receptor and/or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists have been recently proposed as putative ADs. For instance, the NMDA receptor antagonist ketamine is widely used nowadays when patients condition requires an immediate improvement of symptoms, as in presence of suicidal ideations (Shin and Kim 2020).

2.1.2. The role of genetics in Major Depressive Disorder

Genetics is a key factor in the development of MDD. Indeed, initial evidence showed that first-degree offspring of MDD patients had a two to three fold increased risk of MDD and twin study reported a heritability component in MDD of about 37% (Sullivan, Neale, and Kendler 2000). More recently, the involvement of genetics in MDD was confirmed by large Genome-Wide Association Studies (GWASs), although the estimated Single Nucleotide Polymorphism (SNP)-based heritability was lower (8.9%) than that observed in twin studies (Howard et al. 2019). This "heritability gap" had been imputed to several factors that usually are not deeply explored in GWASs, including gene-environment interactions, epigenetic mechanisms, influence of many loci with small effect or, contrarily, of rare variants with larger effect (van Calker and Serchov 2021).

Early studies attempted to elucidate genetic bases of MDD through an "a-priori" approach, namely focusing on genes with a well-known role in pathophysiology of MDD, such as genes involved in the serotonergic and glutamatergic pathways, neurotransmission regulators, neurotrophins, and apoptosis. Recently, a review summarized all the candidate gene studies (n = 141) published between July 2012 and March 2019, revealing a total number of 172 polymorphisms significantly associated with MDD in 85 genes (Norkeviciene et al. 2022). However, only 13 SNPs were confirmed by at least two studies. The lack of replication has been imputed to several limitations observed in these publications, such as a small sample size, no matching or adjustment for gender and/or age, no correction for multiple testing, and high heterogeneity among the studies in terms of population, selection criteria for participants, and genotyping analyses. Moreover, when candidate genes associated variants been evaluated in wide samples (approximately from 62,000 to 440,000 individuals), no association has been confirmed, thus proving a lack of contribution of these variants in MDD onset (Border et al. 2019).

With the advent of GWASs, research moved towards a genome-scale exploration based on a "hypothesis-free" approach across the entire genome, thus also including those genes not previously involved in MDD. Although the first GWASs investigated cohorts too small to detect any significant locus, with the recruitment of a higher number of patients a first SNP in the *PCLO* gene, encoding the Piccolo Presynaptic Cytomatrix protein, was found as associated with MDD and replicated in other studies (Sullivan et al. 2009; Howard et al. 2019; Hek et al. 2009).

The probability to find loci passing the genome-wide significance threshold can also be increased by the meta-analysis of multiple cohorts (Kendall et al. 2021). Indeed, thanks to an adequate sample size (> 1.2 million of individuals) obtained by combining samples from previous studies, one of the last GWASs was able to identify more than two hundred SNPs significantly associated with MDD (Levey et al. 2021).

A useful method to summarize GWAS results in a single value able to explain the whole genetics background of MDD is given by the polygenic risk score (PRS). PRSs can be considered more informative than single SNP associations, since they provide a cumulative representation of the impact of SNPs on disease risk by taking into account all allelic variants associated with the disease, weighted by their effect size. A meta-analysis of MDD GWASs was used to devise a MDD PRS able to explain between 1.5% and 3.2% of phenotypic variance in three different MDD cohorts (Howard et al. 2019). Moreover, an association was found between MDD PRS and different MDD severity degrees when symptoms of the disease were considered as a continuous phenotype (Jermy et al. 2022).

2.2. Treatment Resistant Depression

2.2.1. The challenge of Treatment Resistant Depression

Despite numerous effective medications are available to treat MDD, most patients do not respond adequately to the first pharmacological treatment. Among these, about 30% do not achieve full remission even after multiple treatment attempts and, in such case, are defined as suffering from Treatment Resistant Depression (TRD) (Zhdanava et al. 2021). Although several definitions of TRD have been proposed and applied in the literature, there is a general consensus in defining this condition as the failure of at least two adequate trials with ADs (Voineskos, Daskalakis, and Blumberger 2020).

Therapeutic strategies to manage TRD include both pharmacological and non-pharmacological protocols. Pharmacological treatment strategies are based on several approaches, including switching to another class of drug, combination of different ADs, and introduction of psychotherapy, of a second medication (such as lithium) or of a second-generation antipsychotic. In the last few years, the main focus of researchers has shifted to novel pharmacological compounds, especially ketamine, a NMDA receptor antagonist that showed a rapid and robust antidepressant effect in presence of severe symptoms (Corriger and Pickering 2019). To date, more than sixty clinical trials focused on elucidating the effects of ketamine and its s-enantiomer esketamine in MDD and TRD are ongoing or have been concluded. Several meta-analyses evaluated the usefulness of ketamine/esketamine in depression and confirmed their high effectiveness in rapidly reducing disease symptoms. In particular, the only meta-analysis including exclusively randomized controlled trials (RCTs) on MDD patients evidenced a reduction in depressive symptoms 2-4 hours after intranasal administration of ketamine/esketamine and the peak of antidepressant effect after 24 hours (An et al. 2021). This efficacy peak was confirmed also in TRD patients suffering from bipolar or unipolar MDD, whereas the highest difference in remission between ketamine and placebo groups was observed 7 days after the administration. When restricted to MDD patients, analyses further confirmed efficacy of ketamine in reducing depression severity within 7 days after administration (Kryst et al. 2020). Ketamine utility in treating MDD/TRD was also supported by the low impact of side effects: indeed, these began shortly after treatment start, but they resolved within 1.5-2 hours (An et al. 2021).

In line with these encouraging results, the intranasal usage of esketamine to treat TRD was approved by the U.S. Food and Drug Administration (FDA) in 2019 (Food and Drug Administration Department of Health and Human Services 2019).

2.2.2. Strategies to find genetic biomarkers for Treatment Resistant Depression: omics era

To date, significant efforts have been made to clarify the role of genetics in AD response, and candidate-gene studies have identified several associations between outcome of pharmacological treatments and genes involved in pathways related to MDD (Chiara Fabbri, Corponi, et al. 2019).
With the introduction of GWASs, a non-hypothesis-driven strategy investigating the whole genome was made possible, thus increasing the probability of finding new possible variants in genes not previously explored. Indeed, considering the low success rate of first-line AD treatments and the evident involvement of genetics in response, this information may provide a useful additional tool to guide clinicians towards a more personalized choice of the drug. However, being TRD a well characterized type of MDD from the clinical point of view, but with a still unclear underlying pathophysiology, investigations on wide cohorts of TRD patients and replication studies are needed. In this context, the application of GWAS strategies may help to better elucidate the biological mechanisms underpinning TRD and to evidence further mechanisms involved in treatment resistance, in a perspective of identifying genetic variants that may predict TRD onset, guide the therapeutic treatment and, possibly, identify new specific therapeutic targets.
So far, only one genetic variant (rs150245813), located in pseudogene *PLD5P1*, has been associated with TRD passing the genome-wide threshold of significance in a meta-analysis of GWAS studies (Qingqin S. Li et al. 2020). Moreover, gene-based analysis on a smaller cohort identified an association between TRD and three genes involved in immune regulation: *LTB*, *LST1*, and *NCR3* (Qingqin S. Li et al. 2020). Another candidate gene for treatment resistance is *CACNA1C*, of which SNP rs10848635 was suggested as associated with TRD in a study combining candidate-gene and GWAS approaches (Chiara Fabbri, Corponi, et al. 2018). Further evidence of nominal associations for other variants in *CACNA1C* supported its putative involvement in TRD (Chiara Fabbri, Corponi, et al. 2018). Significant results also emerged for a gene-set involved in cAMP signalling, known for its role in regulating biological mechanisms and transcription of several genes involved in neuronal processes. However, this was not replicated in a meta-analysis performed on a higher number of samples, that instead revealed an association between TRD and chromatin silencing genes (Chiara Fabbri, Kasper, et al. 2019). Other GWASs focused on TRD reported negative findings for SNPs

(Wigmore et al. 2020; Q. S. Li et al. 2016) and Copy Number Variations (CNVs) (O'Dushlaine et al. 2014).

Significant positive correlations were found between presence of treatment resistance and several personality traits, including neuroticism, schizotypal personality, and mood disorder. These results evidenced an overlap in genetic architecture between these traits and TRD, and suggested that patients having these personality traits are more likely not to respond to ADs (Wigmore et al. 2020).

Despite these attempts to elucidate the genetic architecture of TRD, findings are inconsistent and further efforts are still necessary. Lack of robust and replicated results may be due to several limitations affecting these studies, such as the relatively small sample size and the high complexity of TRD phenotype. Indeed, although TRD is defined as failure in at least two AD trials, this definition is not informative on the number of treatment attempts and does not consider that the outcome of treatments with different AD classes are influenced by specific genetic variants (Q. S. Li et al. 2016; Qingqin S. Li et al. 2020).

2.3. Precision Medicine in Major Depressive Disorder

Currently, pharmacological treatment of MDD follows a traditional "trial-and-error" approach, mainly based on the physician's expertise and on the patient's clinical features. However, only a small percentage of patients achieve total remission after the first pharmacological trial, whereas most of them need multiple attempts before responding adequately to the medication and, as a consequence, they have a higher risk of relapse (Rush et al. 2006).

In the last years the concept of precision medicine emerged in all branches of medicine with the intent to provide the most suitable medication to each patient, according to biological, genetic, and clinical features. In psychiatry in particular, a therapeutic approach based on precision medicine would contribute to a higher probability of response to treatment following ADs' administration, thus saving time and reducing costs as well as the risk of developing side effects (Serretti 2018).

Pharmacogenetics and pharmacogenomics (PGx) aim to understand how pharmacological treatment outcome is influenced by individual genetic variability, in terms of single genetic variants and genomic background, respectively. Because of PGx major role in precision medicine, several consortia and working groups have been established with the intention of translating PGx knowledge into clinical practices through the development of recommendations. Among all the publicly available guidelines, the most comprehensive have been elaborated by the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Royal Dutch Association for the Advancement of Pharmacy-Pharmacogenetics Working Group (DPWG) (Relling et al. 2020; Swen et al. 2011), which include recommendations for more than ninety drugs (Yoon et al. 2020). In particular, CPIC guidelines have become the gold standard resource for the implementation of PGx evidence in clinical practice and are used internationally in many centres and institutions providing PGx testing. Briefly, CPIC guidelines classify each gene-drug pair in seven level of suggestion, depending on the literature consistence regarding each gene-drug interaction, from the upper (genetic information should be used to change drug prescribing) to the lowest level (there are few evidence, clinical actions are unclear, little mechanistic basis, mostly weak evidence, or substantial conflicting data regarding gene-drug interaction) (Relling et al. 2020).

Considering all evidence relating to the involvement of genetics in drug response, in 2020 the FDA has published a table including PGx associations supported by sufficiently robust scientific evidence to suggest the utility of PGx testing before prescribing medications (Rubinstein and Pacanowski

2021). Moreover, in the last two decades the FDA approved almost two hundred drugs with PGx recommendations in their labelling (J. A. Kim, Ceccarelli, and Lu 2021).

To make their interpretation more intuitive and their application in clinical practice quicker, PGx guidelines have been reviewed and aggregated in clinical guideline annotations by the Pharmacogenomics Knowledge Base (PharmGKB), a public database including all the available knowledge about PGx (Whirl-Carrillo et al. 2021). In addition to providing prescribing recommendation for many drugs, PharmGKB curates the creation of variant annotations for each gene-drug association, provides a complete description of pathways involved in drugs response, and summarizes FDA drug labels containing PGx information.

2.4. Pharmacogenetics of antidepressants

2.4.1. Pharmacokinetics of antidepressants

Research on PGx of ADs mainly focused on genes involved in pharmacokinetics broadly speaking, comprising mechanisms of adsorption, distribution, metabolism, and excretion of drugs. Pharmacokinetics of ADs mostly depends on the activity of the two members of the cytochrome P450 (CYP) enzyme family *CYP2D6* and *CYP2C19* which, while representing a small percentage of the total hepatic CYP content, are involved in the metabolism of many drugs used in psychiatry, including ADs (Spina and de Leon 2016).

CYP2D6 and *CYP2C19* are highly polymorphic, with 141 and 36 identified haplotypes, respectively (PharmGKB 2021b; 2021a). In order to discriminate each haplotype, these are named using the "star" nomenclature, which associates a "star allele" to each specific combination of genetic variants (e.g., *1 for the reference haplotype) (Robarge et al., 2007). The genetic complexity of *CYP2D6* and *CYP2C19* strongly affects enzymatic activity of their products and can modulate the response to ADs. Indeed, depending on their enzymatic activity, *CYP2D6* and *CYP2C19* haplotypes may be classified as having no function, decreased function, normal function, or increased function. An activity score (AS) was assigned to each haplotype and may be summed to obtain the diplotype AS, which is then used to assign an individual metabolizer phenotype. Thus, depending on their diplotype AS, each individual can be defined as a poor metabolizer (PM; no enzymatic activity), an intermediate metabolizer (IM; decreased enzymatic activity), a normal metabolizer (NM; normal enzymatic activity), or an ultrarapid metabolizer (UM; increased enzymatic activity) (Figure 1). Specifically for

CYP2C19, the additional class of rapid metabolizer (RM; increased enzymatic activity) was defined for those haplotypes with an activity that ranges between NM and UM.

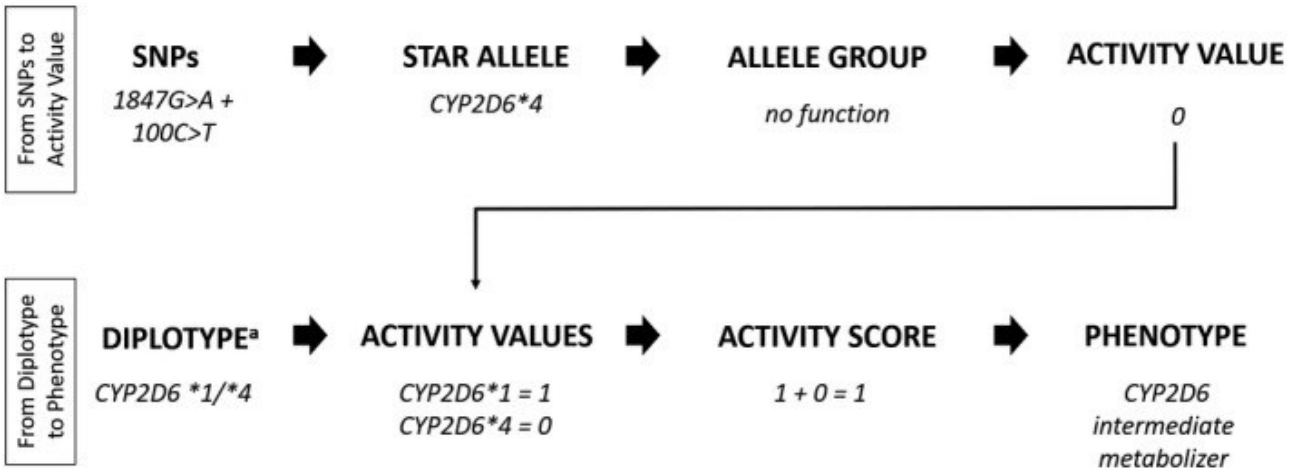

Figure 1: Example of phenotype assignment for *CYP2D6* gene. Briefly, combination of specific genetic variants defines the haplotype (or star allele) of each copy of the gene. Depending on the impact of single haplotype on enzymatic activity, an activity value is attributed to each allele. A total activity score is then obtained by summing the activity value of each allele, thus providing the final individual metabolizer phenotype (Botton et al. 2021).

Some discrepancies complicating the application of a standardized procedure in clinical laboratories offering PGx tests have been observed in *CYP2D6* and *CYP2C19* genotype-phenotype translation system between different guidelines. For this reason, a harmonization of CPIC and DPWG guidelines have been performed by PGx experts and a uniform system for *CYP2D6* genotype-phenotype translation has been established (Caudle et al. 2020). However, incongruities between CPIC and DPWG guidelines for *CYP2C19* metabolizers phenotype classification still exist.

Knowing patient's metabolizer status can help in predicting AD response before administration, thus reducing the risk of providing an unsuccessful medication. Indeed, patients with a PM/IM *CYP2D6* and/or *CYP2C19* phenotype and treated with an AD metabolized by these enzymes are more likely to show a slow drug clearance and, as a consequence, an increased side effect rate due to higher levels of drug in blood (Milosavljević et al. 2021). On the other side, RM/UM phenotypes can more likely lead to a failure of the treatment caused by a higher clearance of ADs (Jukić et al. 2018; Jukic et al. 2019).

Differences in distribution of *CYP2D6* and *CYP2C19* star alleles across Europe further highlighted the need for PGx tests in clinical practice. Indeed, a very heterogeneous frequency of clinically relevant haplotypes have been observed among European populations, also influencing frequencies of metabolizer phenotype classes. For instance, *CYP2D6* duplication, which is associated with an

increased enzymatic activity, showed the lowest frequencies in Northern Europe and was the most frequent in South-Eastern Europe. On the contrary, loss-of-function alleles *CYP2D6*4* and *CYP2D6*5* were the most prevalent in Northern and Central Europe and the least observed in Southern Europe. Similar distributions have been also found for *CYP2C19*: *CYP2C19*17*, which is responsible for an increase in enzymatic activity, was the most common in Central Europe and the rarest in Southern Europe, whereas *CYP2C19*2* was the most prevalent in Northern-Western Europe and the rarest along the Mediterranean coast (Petrović, Pešić, and Lauschke 2020). Furthermore, the probability of having a non-normal metabolizer phenotype, and therefore of not to respond as expected to ADs, was estimated to be 36.4% and 61.9% for CYP2D6 and CYP2C19 enzymes, respectively (Koopmans et al. 2021). The high heterogeneity observed in haplotypes and metabolizer phenotypes distributions across the world further supports the usefulness of PGx tests in clinical practice and the need for guidelines to support clinical decision-making process. To date, CPIC and DPWG provided PGx recommendations for SSRI and TCAs administration based on *CYP2D6* and *CYP2C19* metabolizer phenotype. Although differences can be observed between guidelines, it is universally advised to avoid the administration of an AD to patients with reduced or increased metabolism for that specific medication. When switching to another AD is not possible, guidelines recommend to increase or decrease the drug starting dose accordingly (Hicks et al. 2015; 2017; Brouwer et al. 2021).

2.4.2. Pharmacodynamics of antidepressants

Although PGx tests mainly include genes involved in AD pharmacokinetics because of their well-known effect on drugs and availability of public guidelines, also some pharmacodynamics-related genes have been investigated for their role in AD response and could be considered as a further target for PGx (Shalimova et al. 2021).

Pharmacodynamics refers to all the biochemical, physiological, and molecular effects of drugs on the body. The most studied AD pharmacodynamics-related gene is *SLC6A4*, which encodes the serotonin transporter 5-HTT. 5-HTT is the main responsible for the reuptake of serotonin from the synaptic cleft and is one of the main targets of several ADs, including the first-line medication class for MDD, namely SSRIs. A 43-bp insertion/deletion polymorphism (long/short allele, L/S), known as serotonin-transporter-linked polymorphic region (5-HTTLPR), is present in the *SLC6A4* promoter region and has been implicated in AD response. Indeed, in patients with European ancestry treated with SSRIs, carriers of L allele (LS or LL) showed a higher response and remission rate than those

homozygous for the short allele (SS) (Ren et al. 2020). These differences in AD response based on 5-HTTLPR alleles could be imputed to an approximate 50% reduction in the expression of *SLC6A4* in presence of the S allele, which has been observed in several *in vitro* experiments. However, this reduction has not been confirmed in the human brain (Iurescia, Seripa, and Rinaldi 2016; Murthy et al. 2010). When similar studies have been conducted in others populations, no association between 5-HTTLPR and therapeutic response have been observed (Ren et al. 2020). These discrepancies may be a consequence of differences in allele frequencies among ethnicities. For instance, S allele carriers and SS homozygotes are more frequent in Taiwanese than in European population (S: 79% vs 42%; SS: 60% vs 22%), while the opposite was observed for the LL genotype (1-13% vs 29-43%) (Iurescia, Seripa, and Rinaldi 2016). However, contrasting results have also been reported in European populations, in which some studies observed a significant association between the S allele and both higher remission and response following long-term treatment (Wilkie et al. 2009).

The aforementioned discrepancies may be imputed, for example, to the presence of a SNP within 5-HTTLPR, rs25531, that could exert an additional effect on *SLC6A4* expression levels. Indeed, patients carrying 5-HTTLPR L allele and A in rs25531 (L_A) showed an increased transcription of serotonin transporter, whereas a reduced expression, similar to those observed in S carriers, has been reported in G carriers (L_G) (Kraft et al. 2005). Also, in white non-Hispanic patients, L_A allele was associated with a reduced risk of side effects to citalopram, but no difference was observed in response rate among the different genotypes (X. Z. Hu et al. 2007). Similarly, no significant association was observed between 5-HTTLPR/rs25531 haplotype and response to escitalopram (Maron et al. 2009). In a Japanese cohort, higher response was observed in L_A carriers treated with fluvoxamine, compared to other genotypes, whereas no difference was observed in patients undergoing treatment with paroxetine (Kato et al. 2013). Moreover, a meta-analysis did not find any association between this triallelic polymorphism and AD response, evidencing the need for further investigations (Ren et al. 2020).

Another variant of interest in *SLC6A4* sequence is the 17-bp variable number of tandem repeats in the second intron (STin2 VNTR). Three main STin2 alleles (STin2.9, STin2.10, and STin2.12, with 9, 10, and 12 repeats, respectively) have been identified and have been suggested to modulate *SLC6A4* transcriptional levels (MacKenzie and Quinn 1999). Studies investigating role of STin2 in regulating AD response focused on STin2.10 and STin2.12, which are the most common alleles. The first studies on Korean patients identified a higher frequency of STin2.12 homozygosis in responder to SSRIs

compared to non-responder (H. Kim and Carroll 2006; Kwan Kim et al. 2000), while others were not able to find any association between STin2 and response to fluvoxamine or development of side effects (Ito et al. 2002; Takahashi et al. 2002). A lack of association was also found in white-non Hispanic individuals treated with SSRIs (Shiroma et al. 2014; Smits et al. 2008). Contrarily to previous observations, the Stin2.12 allele has also been associated with a poor response in Taiwanese patients treated with SSRIs or SNRIs (Kao, Chang, and Lung 2018) and with lack of remission and response following paroxetine/citalopram treatment in European individuals (Wilkie et al. 2009). Although evidence suggests a role of STin2 in AD response, available results are still controversial and further confirmations are needed to corroborate its role in AD pharmacodynamics.

Additional variants investigated for their putative role in AD response are located in several other genes, including serotonergic receptors type 1A and type 2A (*HTR1A*, *HTR2A*), norepinephrine transporter (*SLC6A2*), and monoamine oxidase A (*MAO-A*) (Shalimova et al. 2021). However, the role of most of these is still unclear and more evidence is needed to confirm their involvement in AD response.

2.5. Application of genomic techniques to identify new putative biomarkers for antidepressant pharmacogenetics

2.5.1. Identification of pharmacogenomics target through GWAS approach

Application of GWAS in AD pharmacogenomics allowed to identify associations between treatments outcome and genetic variants/genes independently of an *a priori* hypothesis about their role in drug pharmacokinetics or pharmacodynamics, thus offering the possibility to find new genes and variants involved in AD response.

The first GWASs on AD effects in MDD focused on the risk of developing side effects following drugs administration (Laje et al. 2009; Perroud et al. 2012). Despite no SNP reached the genome-wide significance threshold, suggestive associations were found in white patients between treatment-emergent suicidal ideation (TESI) development and two SNPs, rs11628713 (OR: 4.7, $p = 6.20x10^{-7}$) and rs10903034 (OR: 2.7, $p = 3.02x10^{-6}$), located in *PAPLN* and in 3' untranslated region of *IFNLR1*, respectively (Laje et al. 2009). Also, a suggestive association was observed in European MDD patients between the increase of suicidal ideation and rs11143230 (OR: 1.88, $p = 8.28x10^{-7}$), a SNP located 30 kb downstream the gene encoding guanine deaminase (*GDA*), which is involved in synaptic formation and dopamine/glutamate signalling (Perroud et al. 2012). Other SNPs were found to be suggestively associated with TESI risk when patients were stratified for AD classes. For instance, TESI risk in patients treated with nortriptyline was associated with rs6812841 ($p = 7.70x10^{-6}$), located in an uncharacterized locus, whereas in patients undergoing escitalopram treatment the risk of TESI was associated with rs358592 (OR: 0.04, $p = 2.50x10^{-6}$) and rs4732812 (OR: 0.04, $p = 3.35x10^{-6}$), located in *KCNIP4* and *ELP3*, respectively (Perroud et al. 2012). Further variants were associated with citalopram-induced side effects in an ethnically heterogenous sample including about 1,700 MDD patients. In particular, development of vision and/or hearing side effects was significantly associated with a SNP located in *EMID2* (rs17135437, q = 0.03), a gene encoding protein collagen alpha 1 chain, which has been previously associated with ataxia in neurodegenerative disorders (Adkins et al. 2012). An association was also found between rs16965962, located in an intragenic region, and development of general side effects (q = 0.10) (Adkins et al. 2012), whereas sexual side effects development was significantly associated with twelve SNPs in MDD patients treated with bupropion (genome-wide significance threshold $q < 0.05$) (Clark et al. 2012). Interestingly, 10 of these SNPs were in the *SACM1L* gene, which encodes an integral membrane protein located in the endoplasmic reticulum and in the Golgi apparatus and is essential for growth

factor signalling. Alterations in the SACM1L protein may lead to disruption in signalling mechanisms and impairment in hormone and neurotransmitter secretion, which may be the cause of the observed sexual side effects (Clark et al. 2012).

Important consortia have been created with the aim of collecting large datasets, thus maximizing statistical power, including the Sequenced Treatment Alternatives to Relieve Depression (STAR*D), the Genome-Based Therapeutic Drugs for Depression (GENDEP), the Munich Antidepressant Response Signature (MARS), the Mayo Clinic Pharmacogenomic Research Network Antidepressant Medication Pharmacogenomic Study (PGRN-AMPS), and the International SSRI Pharmacogenomics Consortium (ISPC) (Rush et al. 2004; Biernacka et al. 2015; Hennings et al. 2009; Mrazek et al. 2014; Uher et al. 2009).

Initially, even most studies performed on these cohorts did not find any association with a significance that reached the genome-wide threshold, although some interesting results were observed. The first GWAS including STAR*D patients found suggestive associations ($p < 10^{-5}$) between three SNPs and citalopram effects (rs6966038, response: OR: 1.64, $p = 4.65 \times 10^{-7}$, remission: OR: 1.68, $p = 3.65 \times 10^{-7}$; rs6127921, response: OR: 0.61, $p = 3.45 \times 10^{-6}$, remission: OR: 0.57, $p = 1.07 \times 10^{-6}$; rs809736, response: OR: 1.52, $p = 8.19 \times 10^{-6}$) (Garriock et al. 2010). A similar result was observed when STAR*D data were merged with those of about 1,800 European patients treated with SSRI or SNRI in a meta-analysis including about 2,300 individuals (Tansey et al., 2012). No genome-wide association was found even considering AD response as outcome in a multi-ethnic STAR*D cohort, or when the top 25 SNPs detected from this analysis were replicated in the European GENDEP samples (Hunter et al. 2013).

Also in PGRN-AMPS cohort no genetic variant was associated with SSRI outcomes at the genome-wide significance threshold (Ji et al. 2013). However, some of the top SNPs were located in genes of interest for AD response. Indeed, the SNP with the most significant association for the response at 8 weeks of treatment, rs11144870 (OR: 0.42, $p = 1.04 \times 10^{-6}$), is in the gene encoding for riboflavin kinase (*RFK*), an enzyme involved in riboflavin assimilation. Whether overexpressed, riboflavin kinase might lead to insufficient riboflavin levels, previously associated with depressive symptoms. Other suggestive associations were observed between remission at 8 weeks and SNPs proximal to *HTR1B* (rs1379887, OR: 0.49, $p = 9.05 \times 10^{-6}$) and *GRK5* (rs915120, OR: 0.50, $p = 1.15 \times 10^{-5}$), known for their involvement in psychiatric phenotypes (Ji et al. 2013).

A first positive result was observed when three cohorts of European MDD patients from STAR*D, MARS, and GENDEP were meta-analysed in a very extensive study aimed at finding loci associated with remission and symptom improvement at 2 and 12 weeks of treatment. In a cohort of about 2,200 MDD patients, a SNP located in the gene encoding myosin 10 (*MYO10*) showed a significant association at genome-wide level with percentage symptom improvement at 12 weeks (rs17651119, OR: 0.31, $p = 1.78 \times 10^{-8}$), whereas rs12054895, located in an intergenic region, was associated with outcomes at 2 weeks of treatment ($p = 2.65 \times 10^{-8}$). However, these associations were not confirmed by re-genotyping (Uher et al. 2013). Negative results were also found in a meta-analysis of data about 2,400 MDD patients from PGRN-AMPS, STAR*D, and a discovery cohort of about 900 subjects (Biernacka et al. 2015). Although no SNP reached the genome-wide threshold of significance, interesting results emerged from this study. One SNP in particular among those with strongest evidence of association with SSRI treatment outcome, rs10954808 (OR: 0.73, $p = 1.20 \times 10^{-6}$), should be taken in consideration for further investigations. Indeed, rs10954808 is located in the gene coding neurogeulin-1 (*NRG1*), a neurotrophic factor involved in neural maturation and previously associated with risk for mental disorders (Biernacka et al. 2015).

Even the most recent GWAS, which included 10 different European cohorts (including STAR*D, GENDEP, and PGRN-AMPS) and three East Asian cohorts, was not able to identify any genome-wide significant association with AD response in both populations (Pain et al. 2021). However, gene-based analysis on patients with European ancestry (n = 5,000) identified a significant association for *ETV4* and *DHX8* with remission (FDR-corrected p = 0.02) and/or symptoms improvement (*ETV4*, FDR-corrected p = 0.05). Interestingly, both the genes could be implicated in AD response because of their physiological role: indeed, *ETV4* was observed to mediate BDNF-induced hippocampal dendrite development and plasticity, whereas *DHX8* was involved in splicing of messenger RNA (Pain et al. 2021).

Almost all GWASs on AD outcome are focused on response and remission to treatments and do not consider other phenotypes, such as patients Quality of Life (QoL) or personality traits. Only a study in the STAR*D sample showed a suggestive association between rs520210, in *NEDD4L*, and AD response when QoL was considered as an interaction factor (OR: 0.76, $p = 3.64 \times 10^{-8}$) (Antypa, Drago, and Serretti 2014). When QoL was instead included as a model covariate, rs520210 did not reach the genome-wide threshold of significance, but it remained the SNP characterized by the strongest association with AD response (OR: 0.66, $p = 7.01 \times 10^{-7}$). Interestingly, *NEDD4L* encodes the NEDD4

Like E3 Ubiquitin Protein Ligase, which is involved in ubiquitination and protein recycling. This result is in accordance with another GWAS on STAR*D cohort that found rs6966038, a SNP mapped in another ubiquitin ligase (*UBE3C*), suggestively associated with AD response (Garriock et al. 2010).

As it has been done for QoL, also the influence of personality traits (such as extraversion, agreeableness, conscientiousness, neuroticism, and openness) in AD response was assessed using the PGRN-AMPS and the ISPC cohorts (Amare et al. 2018). From these studies a significant association between eight loci, AD response, and some personality traits emerged. In particular, one locus, rs3825243, located near *YEATS4*, was significantly associated with both SSRI response and conscientiousness, whereas seven loci were associated with both remission and neuroticism (rs2979204, rs11990063, rs35792458, rs12555870, rs4761545, rs144733372, rs11082011, located in or near *PRAG1, MSRA, XKR6, ELAVL2, PLXNC1, PLEKHM1,* and *BRUNOL4*, respectively; genome-wide significance threshold $p < 0.05$). Furthermore, PRSs for all the personality traits were calculated in order to evaluate multi-loci impact on AD response. Interesting associations were observed between SSRIs treatment response and/or remission after 4 weeks of treatment and PRSs of conscientiousness, neuroticism, and openness.

As described above, negative and non-reproducible results were observed also when large cohorts of patients were investigated. This may be imputed to a number of additional factors that are not directly related to sample size, including the differences in genetic associations among AD classes. An investigation including about 800 MDD patients with European ancestry from GENDEP study found an association at the genome-wide significance level between a SNP located in *UST* gene, which encodes for uronyl 2-suplhotransferasi, and AD response only in patients treated with nortriptyline (rs2500535, $p = 3.56 \times 10^{-8}$) (Uher et al. 2010). Interestingly, UST enzyme is involved in the production of oversulfated proteoglycans, that are essential for neurogenesis and neuronal migration mechanisms. More recently, the Combining Medications to Enhance Depression Outcome (CO-MED) trial focused on the role of MDD patients' genetic background in modulating response to different pharmacological treatments, including escitalopram monotherapy, escitalopram + bupropion, and venlafaxine + mirtazapine (Gadad et al. 2018). Despite the negative results across all groups, a suggestive association was reached only between treatment response at 6 weeks and rs10769025 (*ALX4*) in MDD patients undergoing escitalopram monotherapy ($p = 9.86 \times 10^{-8}$). Since several additional SNPs located in *ALX4* showed a suggestive association with citalopram response, authors performed haplotype analysis, which revealed a significant association between a specific

haplotype and escitalopram responsiveness after 6 weeks of treatment (OR: 3.4, $p = 2.00x10^{-4}$). Interestingly, the role of *ALX4* in AD response was further confirmed by pathway analysis, which showed an interaction between *ALX4* and several proteins previously implicated in AD treatment outcome, such as COMT, SLC6A4, and HTR2A (Gadad et al. 2018).

Also a meta-analysis of STAR*D and GENDEP cohorts including about 2,000 patients confirmed the differences in AD classes in GWAS analyses (C. Fabbri et al. 2018). Indeed, when several ADs were included in the analysis, no genome-wide significant association was identified. On the contrary, when only patients treated with citalopram or escitalopram were considered (n = 1,739), a significant association was observed between rs116692768 and rs76191705 and symptom improvement after 12 weeks of treatment ($p = 1.87x10^{-8}$ and $p = 2.39x10^{-8}$, respectively). Interestingly, rs116692768 and rs76191705 are located in *ITGA9* and *NRXN3*, two genes that encode proteins previously associated with AD response and synaptic differentiations (C. Fabbri et al. 2018).

Recently, an extensive evaluation of several AD response phenotypes has been performed in patients treated with SSRIs, SNRIs, or NDRIs and enrolled in "Antidepressant Efficacy" (AES, approximately 56,000 participants) and "Antidepressant Efficacy and Side Effect" surveys (AESES, approximately 48,000 participants) (Qingqin S. Li et al. 2020). This study found a genome-wide significant association between rs4955665 and SNRIs response in AES + AESES meta-analysis (OR: 1.25, $p = 1.62 \times 10^{-9}$) and an association between rs4884091 and SSRI response in AES cohort (OR: 1.21, $p = 2.42x10^{-8}$), further confirmed in the merged meta-analysis. Interestingly, two SNPs previously associated with symptom improvement at 12 weeks (rs76191705 and rs116692768) (C. Fabbri et al. 2018) were found associated with NDRIs response at nominal level in AESES cohort ($p = 0.02$ and $p = 0.01$, respectively). Important results also emerged from the pathways analysis, that revealed an enrichment in genes involved in interleukin signalling in SSRIs response and in GABAergic neurotransmission in SNRIs response (Qingqin S. Li et al. 2020).

An additional important factor potentially influencing genetic studies' results is ethnicity. In order to increase the probability of finding positive associations, GWASs should be conducted ideally on cohorts of patients with a shared genetic background, and this is not easy to achieve when analysing large cohorts comprised of patients from multiple centres worldwide. Moreover, because of differences in populations' genetic background, results obtained in a specific ethnic group may not necessarily apply to other populations. For this reason, evidence obtained from previously mentioned GWASs in European populations cannot always be generalized to others, in spite of the

existence of genes and pathways associated with AD response in both European and Asian cohorts (Cocchi et al. 2016).

Most of the GWAS investigating AD response in extra-European patients were conducted on Asian populations, mainly in Chinese and Korean cohorts, and initially did not report significant results (Sasayama et al. 2013). These negative findings could be imputed to the very small samples size and to the heterogeneity in AD classes administrated. Indeed, when a MDD cohort of about 500 Korean patients treated exclusively with SSRIs was evaluated, two SNPs in perfect linkage disequilibrium, rs7785360 and rs12698828, were found associated with AD response ($p < 1x10^{-5}$) (Myung et al. 2015). Results were replicated with a significance reaching the genome-wide threshold in a sample of 230 patients and in the combined cohort of about one thousand patients ($p = 1.60x10^{-8}$ and $p = 6.60x10^{-10}$, respectively). The same association was also found in a non-SSRI (mirtazapine) treated cohort (Bonferroni's correction, p = 0.004) (Myung et al. 2015). Interestingly, rs7785360 and rs12698828 are located in the *AUTS2* gene, which encodes a nuclear protein expressed in the central nervous system and that is widely implicated in neurodevelopmental disorders (Myung et al. 2015).

Lastly, a GWAS focused exclusively on exonic variants was able to find a significant association (exome-wide significance $p = 1.98x10^{-6}$, FDR-corrected p = 0.05) between exm-rs1321744 and AD remission, although the population investigated was relatively small (n = 65). Curiously, exm-rs1321744 is located in a brain methylated DNA immunoprecipitation sequencing site and could be involved in neuronal gene expression regulation through epigenetic mechanisms (Wong et al. 2014).

2.5.2. Role of Polygenic Risk Score (PRS) in predicting AD treatment outcome

As previously observed by Amare and colleagues (Amare et al. 2018), PRS can be useful in estimating genomic influence in AD response phenotypes, thus providing an important value to all those SNPs that do not reach genome-wide threshold of significance but are suggestively associated with AD treatment outcomes. Similarly to single GWASs, also PRS studies showed contrasting results. One of the first PRSs calculated on the basis of a meta-analysis of symptoms' improvement and remission in a GENDEP + MARS cohort was able to predict treatment outcome in STAR*D cohort, explaining between 0.5% and 1.2% of the observed variance (Uher et al. 2013). However, when PRSs were devised separately in the GENDEP and STAR*D cohorts and applied in the other, no significant prediction of AD efficacy was obtained (García-González et al. 2017; Hunter et al. 2013). Finally, when PRSs for MDD and for neuroticism were calculated and tested for a possible association with AD response in a cohort of about 800 European MDD patients, no association overcame the

correction for multiple testing, although results suggested that higher PRS for MDD and neuroticism were associated with less favourable response to SSRIs (Ward et al. 2018).

2.5.3. Application of next-generation sequencing in pharmacogenomic studies

Despite the number of studies and the gradual increase in sample size, results from GWASs are still inconsistent and further investigations are warranted to better understand the role of MDD patients' genetic background in AD treatment outcome. Another useful tool that can be used to identify new genome-wide targets for AD pharmacogenomic studies is given by next-generation sequencing techniques, such as whole-exome (WES) and whole-genome (WGS) sequencing. Contrarily to GWAS, which is mainly focused on common preselected variants included in the array, WES and WGS provide the chance of analysing all nucleotides in the coding portion of the genome or in its entirety, respectively, thus offering the possibility to capture all genetic variations (common as well as rare).

The first WES study on AD response was focused on SSRIs outcome after 12 weeks of treatment in an Estonian MDD sample (n = 10) (Tammiste et al. 2013). 38 SNPs were identified as statistically associated with response to ADs, but only one, rs41271330, was significantly replicated in two larger cohorts ($p < 0.001$). Interestingly, rs41271330 is located in the gene encoding the BMP5 protein, widely expressed in nervous systems and essential for dendritic growth and synapses formation (Tammiste et al. 2013). More recently, another WES study was performed on a MDD cohort of 1,000 Korean patients, revealing a significant association between four SNPs (rs1800014 in *PRNP*, OR: 3.78, p = 0.01; rs6267 in *COMT*, OR: 2.83, p = 0.01; rs200565609 in *BRPF3*, OR: Inf, p = 0.01; rs3213633 in *SLC25A40*, OR:3.27, p = 0.02) and poor early improvement at 2 weeks followed by non-remission at 12 weeks (Kang et al. 2020). Presence of poor early improvement and final non remission was also associated with several pathways and genes involved in mechanisms related to MDD and AD response, including neuronal maintenance, neurotransmitter clearance and metabolism, and inflammatory and epigenetic mechanisms (Kang et al. 2020). Significant results were also obtained in a WES study on a Chinese population that tried to identify genetic variants associated with response in MDD patients undergoing SSRIs monotherapy (n = 530) and in patients undergoing SSRIs + repetitive Transcranial Magnetic Stimulation (rTMS) (n = 399) (Xu et al. 2020). Four SNPs were significantly associated at genome-wide level with SSRIs response (rs3783553 and rs3783550 in *IL1A*, rs11671393 in *GNA15*, and rs4733201 in *PPP2CB*; $p < 5x10^{-8}$), whereas three SNPs

were associated with response to SSRIs + rTMS treatment (rs2303744 in *PLA2G4C*, rs9628662 and rs12034326 in *GBA*; $p < 5x10^{-8}$) (Xu et al. 2020).

Recently, a WGS analysis was conducted on 100 Korean MDD patients treated with escitalopram (Park et al. 2021). Even though no results reached the genome-wide significance threshold, a total number of 36 variants showed a suggestive association with response or remission ($p < 1x10^{-5}$). When four of these variants were investigated in a cohort of about 550 patients treated with SSRIs, association with remission was confirmed only for rs3213755 (OR: 1.75, p = 0.003) (Park et al. 2021). Although the gene in which rs3213755 is located, *KRTAP1-1*, was not previously involved in MDD or AD response, its co-expressed gene, *NTF3*, is essential for neuronal development and hippocampal plasticity, and was found overexpressed in the brain of patients treated with ADs. This evidence might suggest a role for *KRTAP1-1* and rs3213755 in AD outcome, which should be further investigated (Park et al. 2021).

2.6. Application of pharmacogenetics in MDD management

2.6.1. Development of PGx algorithms to guide MDD treatment

The consistent evidence regarding the involvement of pharmacokinetics- and pharmacodynamics-related genes in response to ADs supports the possibility of exploiting PGx knowledge into clinical practice to guide physicians in therapeutical choice process. In this respect, in the last two decades numerous PGx tests investigating genes involved in drugs' metabolic pathways have been developed and commercialized (Table 1).

Name	Producing company	Included genes
AmpliChip (Jain, 2005, p. 450)	Roche	CYP2D6 and CYP2C19
Genefolio (Avera, 2018)	AIHG's pharmacogenomics	17 genes (not otherwise specified)*
Healthspek PGT (Healthspek, 2018)	Healthspek	ABCB1,CYP2C19, F2, MTHFR, ABCG2, CYP2C9, F5, NR1H3, ADRA2A, CYP2D6, GNB3, OPRM1, ADRB1, CYP3A4, GRIK4, RYR1, AGT, CYP3A5, HTR1A, SLC6A2, CACNA1C, DPYD, HTR2A, SLCO1B1, CES1, DRD1, HTR2C, TPMT, CFTR, DRD2, IFNL3, VKORC1, COMT, DRD3, KCNIP1, CYP1A2, EDN1, LDLR*
Millennium PGT (Millennium Health, 2018)	Millennium Health	CYP2C19, CYP2D6, MTHFR
DNA4LIFE (DNA4LIFE, 2018)	DNA4LIFE	CYP1A2, CYP2B6, CYP2C19, CYP2C9, CYP2D6, CYP3A4, CYP3A5, VKORC1, OPRM1, SLC6A4, SLCO1B1*
MyDNA (MyDNA, 2018)	MyDNA	CYP2C19, CYP2C9, CYP2D6, CYP1A2, CYP3A4/A5, VKORC1, SLCO1B1*
GeneSight (Assurex, 2018)	Assurex	CYP2D6, CYP2C19, CYP1A2, CYP2B6, CYP2C9, CYP3A4, SLC6A4, HTR2A
Genecept (Genomind, 2018)	Genomind	SLC6A4, CACNA1C, ANK3, 5HTR2C, MC4R, DRD2, MTHFR, BDNF, YP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4/5
CNSDose (CNSDose, 2018)	CNSDose	ABCB1, ABCC1, CYP2C19, CYP2D6, UGT1A1
Youscript psychotropic (Genelex, 2018)	Genelex	CYP2D6, CYP2C9, CYP2C19, CYP3A4/A5, CYP1A2, SLC6A4, HTR2A
Neuropharmagen (AB-Biotics SA, 2018)	AB-Biotics SA	ABCB1, AKT1, BDNF, CACNG2, CES1, COMT, CRHR1, CYP1A2, CYP2B6, CYP2C19, CYP2C9, CYP2D6, CYP3A4, DDIT4, DRD3, EPHX1, FCHSD1, GRIK2, GRIK4, HLA-A, HTR1A, HTR2A/2C, LPHN3, NEFM, OPRM1, RGS4, RPTOR, SLC6A4, UGT2B15
Mental Health DNA Insight (Pathway genomics, 2018)	Pathway genomics	CYP1A2, CYP2C19, CYP2D6, DRD2, HLA-B, HTR2A/2C, SLC6A4, UGT1A4*
RightMed (OneOme, 2018)	OneOme	CYP1A2, CYP2B6, CYP2C19, CYP2C9, CYP2D6, CYP3A4/A5, GRIK4, HTR2A
BiogeniQ (BIOGENIQ, 2018)	BIOGENIQ	CYP2C19, CYP2C9, CYP2D6, CYP2B6, POR
Antidepressant panel (Quest Diagnostics, 2018)	Quest Diagnostics	CYP2D6
INFINITI CYP2C19 Assay (AutoGenomics, 2018)	AutoGenomics	CYP2C19
Drug-gene testing (Mayo Clinic, 2018)	Mayo Clinic	CYP2D6, CYP2C19
STA2R (SureGene and PGxl, 2018)	SureGene and PGxl	SULT4A1, CYP2D6, CYP2C9, CYP2C19, CYP1A2, CYP3A4, CYP3A5, SLC6A4, MTHFR*
Pharmacogenetic testing (LabCorp, 2018)	LabCorp	CYP2D6, CYP2C9, CYP2C19, CYP1A2, SLC6A4, HTR2A/C*
TreatGx (GenXys, 2018)	GenXys	> 60 genetic markers in genes including CYP2C19, CYP2C9, CYP2D6, VKORC1, G6PD, HLA-A, HLA-B, SLCO1B1*
HILOmet (Genomas, 2018)	Genomas	CYP2D6, CYP2C9, CYP2C19
Rxight (MD labs, 2018)	MD labs	ANKK1, ADRA2A, COMT, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP3A4/A5, DPYD, GRIK4, HTR2C, MTHFR, OPRM1, SLCO1B1, TPMT, UGT2B15, VKORC1
GeneAlign (GeneAlign, 2018)	GeneAlign	19 genes associated with the metabolism, response and interactions (not otherwise specified)*
RenaissanceRX (RenaissanceRX, 2018)	RenaissanceRX	CYP3A4/3A5, CYP2C19, CYP2D6
Antidepressants and antipsychotics pharmacogenetics (CGC Genetics, 2018)	CGC Genetics	CYP2D6, CYP2C19
PGxOne (Admera Health, 2018)	Admera Health	GRIK4, HTR2A/1A, SLC6A4, ABCB1, ADRA2A, CYP2D6, CYP2C19, CYP3A4, CYP1A2
Pillcheck (Geneyouin, 2018)	Geneyouin	CYP2D6, CYP2C19, CYP2C9, CYP3A4/A5, CYP1A2, OPRM1, SLCO1B1, VKORC1*
GeneTrait Psychotropic Panel (GeneTrait Laboratories, 2018)	GeneTrait Laboratories	9 genes (not otherwise specified)*
Pharmacogenetic panel (Bio.logis, 2018)	Bio.logis	COMT, CYP1A2, CYP2C19, CYP2D6, OPRM1, SLC19A1
Pharmacogenetic Screen (Sonic Genetics, 2018)	Sonic Genetics	CYP2D6, CYP2C19
Pharmacogenomic tests (Lab Tests Online, 2018)	Lab Tests Online	CYP2D6, CYP2C9, CYP2C19, CYP1A2, SLC6A4, HTR2A/C*
Pharmacogenetic Psychiatry report (Alpha Genomix, 2018)	Alpha Genomix	CYP2D6, CYP2C9, CYP2C19, CYP3A, CYP1A2
Pharmacogenetic testing (Ancillary Medical Solutions, 2018)	Ancillary Medical Solutions	CYP450 genes
Drug metabolism (Vantari Genetics, 2018)	Vantari Genetics	CYP2D6, CYP2C19
Pharmacogenomic test (Aeon Global Health, 2018)	Aeon Global Health	CYP2D6, CYP2C19
PharmacoScan (Thermo Fisher Scientific, 2018)	Thermo Fisher Scientific	CYP2C19, CYP2D6
NeuroIDgenetix (Bradley et al., 2018)	Althea Dx	CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5, SLC6A4, COMT, HTR2A, MTHFR
Pharmacogenetic DNA testing (Medigenetics, 2018)	Medigenetics	CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP3A4/A5, SLCO1B1

Table 1: Available commercial PGx test for depression (Chiara Fabbri, Zohar, and Serretti 2018).

First-generation PGx tests targeted few single genes, such as *CYP2D6* and *CYP2C19*, and did not account for the potential synergic and additive effects of multiple genes on drugs' metabolic pathways. Only development of "combinatorial" PGx tests allowed to provide clinical indications for

each medication based on a weighted and combined assessment of genetic variants in multiple pharmacokinetics- and pharmacodynamics-related genes. Validity of some commercial combinatorial PGx tests has been evaluated by comparing their ability to predict changes in drugs' metabolism with those of single-gene CPIC guidelines. These studies revealed that combinatorial PGx tests were able to correctly predict patients' symptom improvement, response, remission, and medication blood levels, whereas guidelines based on single genes failed in predicting outcome of treatments (Altar et al. 2015; Rothschild et al. 2021).

Most of the commercial combinatorial PGx tests are based on proprietary algorithms and, therefore, the exact process through which genetic data and PGx knowledge are combined into clinical recommendation is not publicly available (Bousman and Eyre 2020). However, as well explained on the GeneSight® website ("The GeneSight® Psychotropic Combinatorial Algorithm | GeneSight" 2022), three main steps are needed for the development of the algorithm that produces PGx reports for clinicians: 1) a comprehensive review of the PGx literature for the selection of the more influencing genetic variants, 2) the genotyping of pre-selected polymorphisms, and 3) the creation of a written report based on the information obtained from the previous two phases (Figure 2).

An extensive review of the PGx literature constitutes the basis of all PGx algorithms and represents the first step for the final report generation. Depending on criteria previously established from each PGx test manufacturer, starting PGx knowledge may include several sources, among which the PharmGKB database, CPIC and DPWG guidelines, and single studies investigating gene-drug interactions. Final guidelines will include suggestion about drug administration and dosage for those genes having strong literature evidence of gene-drug interaction. The constant discovery of new evidence in PGx field and the periodical revision of algorithms' guidelines allow to keep PGx tests up to date, thus increasing the effectiveness of PGx-guide treatments.

Genetic information regarding the pre-selected variants is obtained through genotyping of a patient's DNA sample, usually collected through non-invasive techniques (e.g., buccal brushes). Both pharmacokinetics- and pharmacodynamics-related genes may be included in the algorithm. In order to obtain the patient's overall metabolizer status, enzymatic activity of each pharmacokinetics-related genes is predicted on the basis of gene diplotype and merged in a unique weighted metabolic pathway for each drug. Depending on global enzymatic activity, drugs' metabolism may result as increased, normal, or reduced. Similarly, variants in pharmacodynamics-related genes are genotyped and their individual influence on likelihood of response and risk of side effects are

combined with pharmacokinetics information in order to obtain the overall impact on treatment outcomes.

In the last phase, genetic information and indications coming from PGx guidelines are combined and the results of the PGx test is provided in a written report, which classifies medications into different categories, depending on how genetic background is expected to influence treatment outcome. The major part of reports includes three different categories: a green class, in which those medications that, on the basis of patient's genetic background, may be considered the first therapeutic choice at the standard dose are allocated; a yellow class, for drugs that could be ineffective or induce side effect and, therefore, need for dose adjustment; a red class, for those drugs that have an high probability of treatment failure or to induce side effects and, therefore, are highly not recommended for that patient or need dose adjustment. When a drug is placed in a category of suggestion of gene-drug interaction, clinical considerations are provided in order to explain clinicians the rationale of classification and to eventually specify drug's dosage suitable for the patient.

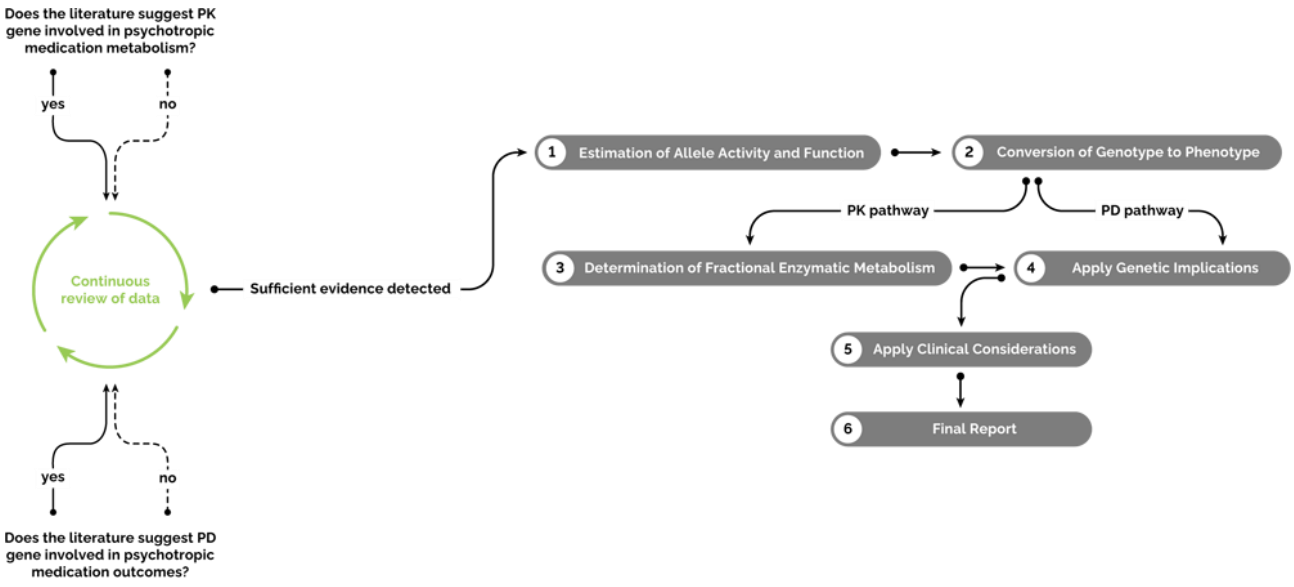

Figure 2: GeneSight algorithm ("The GeneSight® Psychotropic Combinatorial Algorithm | GeneSight" 2022).

Although available PGx tests attempt to include the most recent evidence regarding gene-drug interactions and try to investigate the widest number of genetic variants, potential errors in report generation may occur and they should be taken in consideration when PGx test results are applied in clinical practice to guide pharmacological treatment.

One of the main issues in the reliability of PGx test is the risk of inaccuracy of metabolizer status assignment. To date, individual metabolizer status is obtained through genotyping of the most common genetic variants with a well-known effect on gene-drug interaction. However, some patients could show variants that are not included in the genotyping panel, such as less common genetic variants or unknown variants. Lack of this information during haplotypes definition may result in the assignment of star alleles with a totally different enzymatic activity compared to the actual one. In this scenario, PGx test would potentially output a wrong metabolizer status and, as a consequence, lead to a dangerously incorrect clinical management of the patient.

Also, uncertain metabolizer phenotype identification could be the consequence of unclear haplotype and diplotype definition due to errors in the genotyping process. Indeed, in presence of a *CYP2D6* duplication it could not be clear which copy of the gene is duplicated and, as a consequence, diplotype assignment could be wrong. For instance, a duplication of *CYP2D6* in patients with a **1/*4* diplotype can result in either *CYP2D6*1x2/*4* or *CYP2D6*1/*4x2*, which correspond to an AS of 1 and 2, respectively (Dalton et al., 2020). Further ambiguity could be given by cis and trans allele-specific localization. For example, the two variants 100C > T and 1846G > A can define both *CYP2D6*1/*4* diplotype and *CYP2D6*4M/*10* , depending on their configuration in cis or trans (Yang et al. 2017). However, these two diplotypes correspond to two distinct metabolizer status (NM and IM, respectively) that impact on ADs' metabolism in a different manner.

In all the cases of uncertainty, diplotype is commonly assigned on the base of the prevalence in the specific population, whereas, when no variants are detected, patient is considered to have the wild-type genotype and is predicted to have a normal enzymatic activity. A more precise identification of haplotype would be possible through gene sequencing. However, sequencing is not easily applicable in the clinical practice and could lead to the identification of rare and/or novel star allele with an indetermined or unclear phenotype (Yang et al. 2017). Since, at least to date, whole gene sequencing is not a valid alternative to PGx test in clinical practice, re-testing should be considered an option for those patients who do not benefit adequately from the first PGx test. Indeed, research on AD PGx is a growing field and new variants with a role on enzymatic functions could be included in future PGx tests, thus increasing the probability of identifying patients' metabolizer phenotype with more accuracy.

A further example of misinterpretation regards the assignment of the *CYP2D6* genotype that may be influenced by the highly homologous pseudogene *CYP2D7*, located upstream the *CYP2D6*

sequence in the *CYP2D* locus. Given the high variability of the *CYP2D* locus, fusion of these two genes occurs frequently and can lead to non-functional hybrids. When genotyped, these hybrids may be misclassified as a duplicated *CYP2D6*, thus causing an over-estimation of the metabolizer phenotype, or they may result in an inconsistent genotype (Gaedigk et al. 2010).

Finally, an important issue that PGx tests do not consider is the phenomenon of phenoconversion. Phenoconversion is defined as a mismatch between the metabolizer phenotype predicted from CYP genes genotyping and the real individual metabolizer status. Enzymatic activity of CYP2D6, CYP2C19 and other CYP family members can be influenced by several non-genetic factors, including sex, age, nutritional conditions, hormones, smoking, comorbidities, and concomitant use of medications that may alter ADs' metabolism. Indeed, phenoconversion may occur when, for instance, an inhibitor of CYP2D6 activity is administrated to the patients. Individuals with PM status may be not affected by phenoconversion, because they already have a non-functional enzyme, while in IM patients this can induce a conversion to PM, leading to a lack of functional enzyme which cannot be identified by the PGx test (Klomp et al. 2020). Phenoconversion can explain the conflicting results among studies aiming to evaluate the PGx test application in MDD treatment. Intuitively, when conversion to a metabolizer phenotype different from those identified by PGx test occurs, patients are less likely to respond to a PGx-guided treatment, given that PGx testing is implicitly agnostic to non-genetic factors. Because of its frequency, phenoconversion risk should be considered when a PGx test is administrated. Indeed, in a cohort of MDD patients treated with venlafaxine, the rate of conversion from non-PM to PM due to the concomitant administration of CYP2D6 substrates or enzyme inhibitors has been estimated to be approximately 24% (Preskorn et al. 2013).

2.6.2. Implementation of commercial combinatorial PGx tests in clinical practice

To date, five commercial combinatorial PGx tests have been evaluated for their potential utility in addressing clinicians towards the most effective pharmacological treatment for each MDD patient. In order to assess usefulness of such tests, several trials have compared outcomes of ADs chosen following PGx test suggestions ("PGx-guided" or "Treated with Genetic Test Guide" TGTG group) with those of medications prescribed according to classical clinical practice ("control" or "Treated As Usual" TAU group).

Clinical outcomes of PGx-based AD treatments were evaluated across studies using several clinical scales, such as, for example, the 17-item Hamilton Depression rating scale (HAMD-17), the 16-item Quick Inventory of Depression Symptomatology (QIDS-C16), the Patient Health Questionnaire 9

(PHQ-9), and the Beck's Depression Inventory (BDI). Generally, depressive rating score/symptoms improvement was evaluated as the percentage reduction of HAMD-17 (e.g., at week 8), with remission defined as HAMD-17 ≤ 7/8 (in some studies coupled with QIDS-C16 ≤ 5/6 and PHQ-9 < 5) and response defined as a reduction of at least 50% in HAMD-17 from baseline (Greden et al. 2019; D. K. Hall-Flavin et al. 2012; Bradley et al. 2018; Perlis et al. 2020; Tiwari et al. 2022). One study used BDI to assess symptom improvement (% decrease of BDI score), response (decrease of at least 50% in BDI score), and remission (BDI ≤ 10) (Tanner et al. 2018). Side effect changes have often been evaluated by means of the Frequency, Intensity, and Burden of Side Effects Rating Scale (FIBSERS) (Winner et al. 2013; D. K. Hall-Flavin et al. 2012).

Perhaps the most evaluated commercial kit in MDD PGx literature is the combinatorial GeneSight® Psychotropic test, which initially classified 26 psychiatric medications based on the genotype of 33 known variant sites located in five AD pharmacokinetics- or pharmacodynamics-related genes (*CYP2D6*, *CYP2C19*, *CYP1A2*, *SLC6A4,* and *HTR2A*). The first prospective non-randomized open label trial evaluating the GeneSight® test in a MDD cohort (n = 44) revealed a significant reduction of symptoms across the study in TGTG group compared to TAU patients, which survived correction for multiple testing (31.2% vs 7.2% in QIDS-C16 score, p = 0.002; 30.8% vs 18.2% in HAMD-17 score, p = 0.04)(D. K. Hall-Flavin et al. 2012). Interestingly, an increase in depressive symptom scores was observed in TAU patients between weeks 4 and 8, whereas patients treated with a PGx-guided medication continued to respond adequately to the treatment (D. K. Hall-Flavin et al. 2012). These results were successfully replicated in a larger cohort of patients (n = 227), including both MDD and patients suffering from depressive disorder not otherwise specified (DDNOS) (Daniel K. Hall-Flavin et al. 2013). Moreover, application of GeneSight® test indications was associated with the highest rates of symptom improvement, supporting its utility in clinical decision making. Conversely, TAU patients taking the least suited medication according to their genetic background showed the lowest symptom improvement (Daniel K. Hall-Flavin et al. 2013). Differences in depressive score between TGTG and TAU observed by Hall-Flavin and colleagues were also replicated in a double-blind RCT including 51 patients with MDD or DDNOS, although the number of participants was limited and the observed differences did not achieve significance (Winner et al. 2013).

The Genomics Used to Improve Depression Decisions (GUIDED) study was the first blinded randomized long-term controlled trial evaluating the GeneSight® test in a cohort of MDD patients with a history of AD trial failure (n = 1,541) (Greden et al. 2019). The GeneSight® test evaluated in

the GUIDED trial included three additional genes (*CYP2C9*, *CYP3A4*, and *CYP2B6*), a total number of 59 alleles and variants, and 38 psychotropic medications. In contrast with previously published evidence, no difference in symptom improvement was observed between TGTG and TAU groups after 8 weeks of treatment (27.2% vs 24.4% decrease in HAMD-17, p = 0.11). However, response and remission rates were significantly higher in TGTG than in TAU (response: 26.0% vs 19.9%, p = 0.01; remission: 15.3% vs 10.1%, p = 0.01). Considering the course of MDD throughout the 24 weeks of study, PGx-guided group showed a total decrease in symptom rate of 42.5% and an increase in response and remission rate starting from week 8 (70% and 100%, respectively). Also, the GeneSight® test helped in reducing the mean number and the risk of developing side effects in those patients who switched to a medication prescribed in a genetic background-aware fashion by week 8 (Greden et al. 2019). Contrarily to what was observed in the whole cohort, when only the participants taking medications subject to gene-drug interaction at baseline were considered (n = 912), a significant improvement of symptoms was observed at week 8 in TGTG group, compared to TAU (27.1% vs 22.1% of decrease in HAMD-17, p = 0.03) (Thase et al. 2019).

Results observed in the GUIDED trial were concordant with those obtained from the prospective, open-label "Individualized Medicine: Pharmacogenetics Assessment and Clinical Treatment" (IMPACT) trial (Tanner et al. 2018), aimed at assessing whether outcomes of PGx-guided AD treatment in patients affected with moderate-to-severe MDD (n = 1,871) may change depending on the AD administration setting (primary care providers or psychiatrists). When the whole cohort was evaluated, results confirmed previous evidence about the Genesight® test efficacy. Indeed, independently of the setting, after 8-12 weeks of PGx-guided treatment patients showed a statistically significant reduction of BDI symptoms score (27.9%, p < 0.01) and an increase in response and remission rates of 25.7% and 15.2%, respectively (Tanner et al. 2018).

Recently, the double-blind Canadian Genomic Applications Partnership Program-Major Depressive Disorder (GAPP-MDD) RCT was concluded (Tiwari et al. 2022). The GAPP-MDD trial aimed to investigate the long-period utility of PGx-guided AD treatment in MDD patients who failed at least one pharmacological trial. Beyond those included in the GeneSight® test, GAPP-MDD trial evaluated seven additional variants in six genes known for their role in antipsychotic-induced weight gain (e.g., *MC4R*, *CNR1*, *NPY*, *GCG*, *HCRTR2*, and *NDUFS1*). However, no difference was found between outcomes of the classical and the enhanced GeneSight® tests, and patients were aggregated in a single TGTG group for further analyses. Compared to TAU, at week 8 patients in PGx-guided arm

showed noticeable symptom improvement (27.6% vs 22.7% decrease in HAMD-17), response (30.3% vs 22.7%), and remission rates (15.7% vs 8.3%). Although not statistically significant ($p = 0.27$, $p = 0.26$, and $p = 0.13$, respectively), these results are supportive of those observed in the GUIDED study and they further support application of the GeneSight® test in MDD management (Tiwari et al. 2022; Greden et al. 2019).

Alongside commercialization of the GeneSight® test and investigation of its utility in the clinical setting, further PGx tests have been developed and assessed in MDD, including Neuropharmagen® (NFG®), CNSDose®, NeuroIDGenetics®, and Genecept®. Although only a limited number of studies have assessed the application of these tests in MDD, positive evidence obtained so far have highlighted how their implementation in the clinical practice would contribute to improve the outcome of AD treatments. The NFG® test, which includes 74 variants and alleles in 30 PGx-related genes and classify 59 psychiatric medications, was evaluated in both double- and single-blind RCTs with encouraging results. A lower risk of developing side effects starting from week 6 and a higher response rate at week 12 were observed in 520 MDD Spanish patients treated with a NFG® PGx-guided medication (51.3% vs 36.1%, OR:1.86, $p = 0.01$) (Pérez et al. 2017). Moreover, the NFG® test successfully guided drug prescription also in presence of multiple previous AD trial failures, significantly improved depressive symptoms and reduced side effect scores after 8 weeks of treatment (Han et al. 2018). Positive results in treatment of patients who failed at least one AD trial were obtained also with the Genecept® test, which includes 57 variants in 18 PGx-related genes. Indeed, a randomized double-blind RCT comprising 296 patients revealed a higher probability of remission in patients treated with an AD suggested by the Genecept® test (33.7% vs 18.5%, OR:2.23, $p = 0.01$). However, contrarily to other PGx commercial tests, this was not able to reduce side effect risk (Perlis et al. 2020).

Encouraging results supporting PGx-guided therapy in MDD were also obtained for the CNSDose® and the NeuroIDGenetics® tests, which investigate 5 and 10 PGx-related genes, respectively. Patients treated with a CNSDose®-based therapy showed an increase in remission rate at week 12 compared to control group (OR: 2.52, $p < 0.0001$) and, similarly, the NeuroIDGenetics® test was observed to increase both remission and response rates (remission rate at 12 weeks: 35% vs 13%, OR: 3.52, $p = 0.02$; response rate at 12 weeks: 73% vs 36%, OR: 4.72, $p = 0.001$) (Singh 2015; Bradley et al. 2018). However, contrasting results were obtained when side effect risk was evaluated, in that the NeuroIDGenetics® test was not able to reduce the risk of side effects while the CNSDose®

significantly contributed to increase AD tolerability (OR: 1.13, p = 0.03) (Singh 2015; Bradley et al. 2018).

Despite the aforementioned results widely supported implementation of PGx test in clinical practice to guide MDD treatment, it must be noted that PGx test manufacturers directly funded most of the studies or financially supported the authors, creating a potential conflict of interests. When the efficacy of a non-commercial PGx test including 45 genetic variants in five genes (*CYP2C19*, *CYP2D6*, *CYP1A2*, *SLC6A4*, and *HTR2A*) was evaluated in a Chinese population, no significant differences were found between outcomes of TGTG and TAU groups. Indeed, although response and remission rates in PGx-guided group were higher than those observed in TAU group, differences were not statistically significant (response rate: 74.2% vs 57.5%; remission rate: 61.3% vs 45.0%) (Shan et al. 2019). In addition, both groups showed a statistically significant decrease of HAMD-17 score during the 8 weeks of trial ($p < 0.01$) and no difference was observed in the HAMD-17 score and in HAMD-17 score reduction at each time point. However, these results may be imputed to several factors, including the limited sample size, the presence of patients taking a medication concordant with their genetic background in the control group, or the placebo effect in PGx-guided group given by the patient's knowledge regarding their group allocation.

2.6.3. Economic and social impact of PGx tests

One of the main advantages of the application of PGx tests in the clinical practice is their positive influence on the economic burden of MDD. Overall costs of MDD treatment based on traditional approach has been estimated to be greater than $200 billion, with an annual increment of direct medical cost of approximately $6,400 per patient, that may increase in presence of resistance to treatments (Johnston et al. 2019; Greenberg et al. 2015). Contrarily, when PGx-based ADs are administrated, long term overall costs are reduced. A three years cost-effectiveness study observed that the use of the NeuroIDGenetics® test to guide MDD treatment allowed to save between $2,500 and $6,000, depending on the severity of the disease (Groessl et al. 2018). Similarly, in a Canadian clinical trial PGx testing was estimated to enable savings between $1,687 and $3,056 per patient compared to unguided treatment (Tanner et al. 2020).

Despite the financial advantages brought to the national healthcare by PGx testing, their application in clinical practice is hampered by concerns of clinicians and patients. Doubts regarding how to incorporate PGx tests in the clinical workflow, the waiting time for test results, the lack of knowledge about PGx of ADs, the risk of discrimination due to test results, and more in general the fear of result

misinterpretation keep limiting the use of such tests (Jameson et al. 2021; Vest et al. 2020). However, both patients and clinicians are hopeful that PGx testing would reduce the number of unsuccessful trial attempts, the likelihood of side effects, and the time to find an effective treatment (Jameson et al. 2021; McCarthy et al. 2020).

3. General aim

Pharmacogenetic analysis of AD therapies in MDD has become of great interest in the last decades especially because of the wide spread of the disease worldwide and of the difficulties in treating patients promptly. In spite of the huge efforts carried out by the research community in identifying genetic predictors of non-response, side effects, or adverse reactions to ADs, further investigations are still necessary to translate research results into patient management changes in the clinical practice.

The main purpose of this thesis was to provide additional insights into the role of PGx in treatment of MDD, through both a clinical and a molecular approach, and to better elucidate the genetic underpinnings of TRD.

The first main purpose of this study was to evaluate the usefulness of the potential implementation of PGx in the clinical practice to successfully manage MDD. In particular, the study described in Chapter 4 aimed at the development of a non-commercial and open-source PGx algorithm able to predict, on the basis of up-to-date genetic information and literature guidelines, the best AD treatment for each patient. Moreover, the utility of such PGx test in the clinical practice has been then evaluated through a retrospective analysis in a cohort of MDD patients that have been treated with one or more AD (described in Chapter 5). The aim of this part of the study was to evaluate whether the outcome of pharmacological treatments could be predicted by the PGx algorithm and, therefore, whether PGx testing could contribute to guide clinicians towards an optimal therapeutic choice, reducing both treatment time and cost.

The second main aim of this thesis was to better understand the genetic background underlying TRD analysing the role of pre-selected candidate genes previously associated with AD response. In particular, genetic association studies conducted here were focused on two major aspects: the first one, described in Chapter 6, was centred on members of the CYP family and on *SLC6A4*, known to have a relevant role in AD pharmacokinetics and pharmacodynamics, whereas the second, described in Chapter 7, investigated the potential role in TRD of specific genetic variants proposed as implicated in AD PGx but not characterized by robust associations.

In the last study, described in Chapter 8, we investigated possible alterations of leukocyte telomere length (LTL) in TRD patients in order to assess whether LTL could be involved in the genetic background that underlies resistance to treatment. The final purpose of these studies was to provide

new information for the development of innovative biomarkers for early TRD identification and the consequent optimization of treatment.

4. Development of a combinatorial PGx test to guide therapeutic decision-making in MDD patients

4.1. Aim of the study

The aim of this study was the creation of an independent and up-to-date PGx test supporting clinicians in choosing the optimal AD for MDD patients who did not respond to at least one previous AD pharmacological trial. Three main steps were included in the PGx algorithm: 1. identification of genetic variants involved in PGx of ADs; 2. drafting of PGx guidelines for each gene-drug interaction; 3. creation of a user-friendly PGx report based on patient's genetic background.

4.2. Material and methods

Identification of genetic variants having an impact on AD outcome

Genetic variants included in our PGx algorithm were chosen considering clinical annotations reported in the PharmGKB database. Briefly, for each genetic variant listed in the database, clinical annotations describe the impact of alleles on pharmacological treatment outcome on the basis of clinical guidelines (e.g. CPIC, DPWG), FDA-approved drug labels, and variant annotations. These last are given by each publication reporting an association between a variant and a given drug outcome.

Clinical annotations are classified according to six levels of evidence (1A, 1B, 2A, 2B, 3, and 4), based on the score of supporting annotations (Table 2). Only those genetic variants having a high or moderate clinical annotation level (1A, 1B, 2A, and 2B) for the most used ADs in Italy were included in our PGx algorithm.

Level of Evidence	Description
1A	Level 1A clinical annotations describe variant-drug combinations that have variant-specific prescribing guidance available in a current clinical guideline annotation or an FDA-approved drug label annotation. Annotations of drug labels or clinical guidelines must give prescribing guidance for specific variants or provide mapping from defined allele functions to diplotypes and phenotypes to be used as supporting evidence for a level 1A clinical annotation. Level 1A clinical annotations must also be supported by at least one publication in addition to a clinical guideline or drug label with variant-specific prescribing guidance.
1B	Level 1B clinical annotations describe variant-drug combinations with a high level of evidence supporting the association but no variant-specific prescribing guidance in an annotated clinical guideline or FDA drug label. Level 1B clinical annotations must be supported by at least two independent publications.
2A	Variants in Level 2A clinical annotations are found in PharmGKB's Tier 1 Very Important Pharmacogenes (VIPs). These variants are in known pharmacogenes, implying causation of drug phenotype is more likely. These clinical annotations describe variant-drug combinations with a moderate level of evidence supporting the association. For example, the association may be found in multiple cohorts, but there may be a minority of studies that do not support the majority assertion. Level 2A clinical annotations must be supported by at least two independent publications.
2B	Variants in Level 2B clinical annotations are not in PharmGKB's Tier 1 VIPs. These clinical annotations describe variant-drug combinations with a moderate level of evidence supporting the association. For example, the association may be found in multiple cohorts, but there may be a minority of studies that do not support the majority assertion. Level 2B clinical annotations must be supported by at least two independent publications.
3	Level 3 clinical annotations describe variant-drug combinations with a low level of evidence supporting the association. This association may be based on a single study annotated in PharmGKB, or there may be several studies that failed to replicate the association. The annotation may also be based on preliminary evidence (e.g., a case report, non-significant study, or in vitro, molecular, or functional assay evidence), resulting in a lower calculated score.
4	Level 4 clinical annotations describe variant-drug combinations where the total score is negative, and the evidence does not support an association between the variant and the drug phenotype.

Table 2: Description of level of evidence attribution (from https://www.pharmgkb.org/page/AnnLevels).

The algorithm also included variants without a high or moderate clinical annotation level if these were in pharmacodynamics-related genes with a well-known role in AD response.

Metabolizer status assignment

For each pharmacokinetics-related gene included in the algorithm, the diplotype AS was identified by integrating genotyping data with the allele definition table available in the PharmGKB database, providing information about variants that define star alleles. When more than one diplotype was attributed to a patient, the one with higher frequency in the European population was selected. Finally, metabolizer phenotype was attributed to each diplotype according to the PharmGKB diplotype-phenotype table (PharmGKB 2021a; 2021b).

PGx guideline drafting

PGx guidelines, used by the algorithm to allocate medications in the final report, were created by merging CPIC/DPWG clinical guidelines and variant annotations in order to provide genotype-based dosing recommendations for pharmacokinetics- and pharmacodynamics-related genes, respectively.

A single indication was associated with each gene-AD interaction and a recommended category was attributed depending on its effect on the treatment outcome: “use as first choice” (labelled “Green”), “use with caution” (labelled “Yellow”), and “use with extreme caution” (labelled “Red”).

PGx report generation

Drugs’ allocation was performed with a custom-made R package called *PharmGenBS* that will be freely available at the end of the study. When more than one category of suggestions was attributed to the same AD because of multiple gene-drug interactions, this was placed in the category with more warnings. Medications with no PGx guidelines were included in the “use as first choice” category, and a note regarding the lack of information was added to the report in such cases. The flowchart in Figure 3 summarizes the workflow of the PGx algorithm.

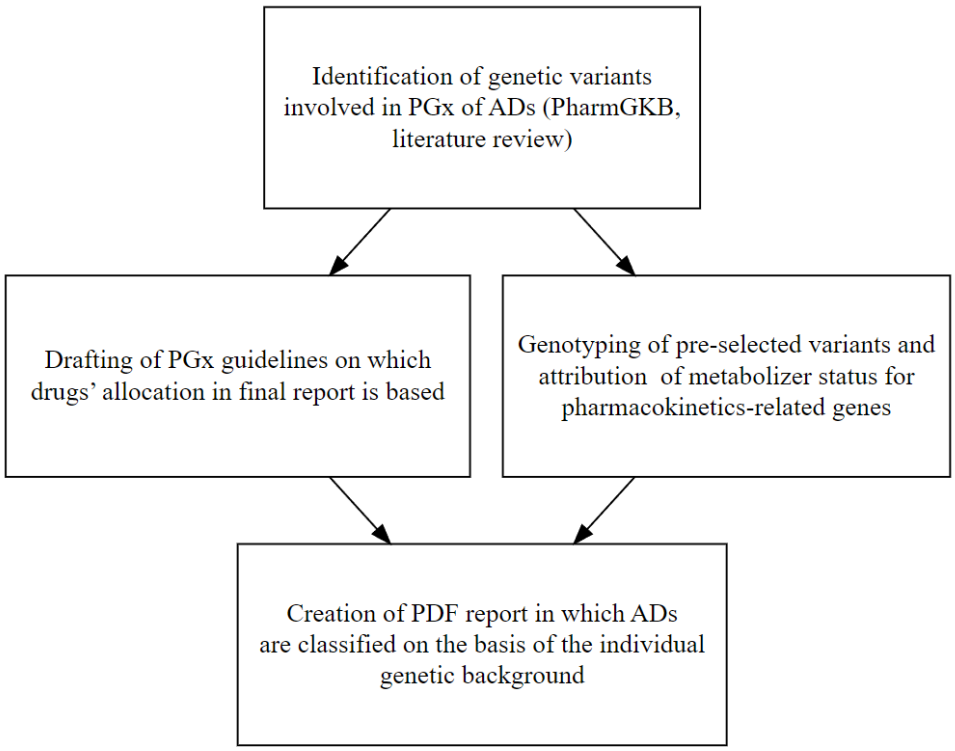

Figure 3: Flowchart of the workflow of the algorithm developed for creating the PGx report.

4.3. Results

Genetic variants included in the algorithm

A total of 26 genetic variants with high or moderate clinical evidence of association with AD response in PharmGKB database and enabling discrimination of *CYP2D6* and *CYP2C19* haplotypes were included in the algorithm. Sixteen variants were in *CYP2D6*, including one CNV and 15 SNPs, whereas 10 SNPs were in *CYP2C19*.

Five further variants located in 5 pharmacodynamics-related genes have been selected based on published evidence because of their role in AD PGx. An insertion/deletion polymorphism, 5-HTTLPR, was in *SLC6A4*, whereas the remaining 4 SNPs were in *FKBP5* (rs4713916), *MC4R* (rs489693), *HTR1A* (rs6295), and *HTR2A* (rs7997012).

For each SNP included in the panel, information regarding type of variant and allele frequency among biogeographic populations are reported in Table 3.

Genetic variant			Allele frequency in biogeographical groups											
SNP	Type of variant	Gene	Global	European	African	African Others	African American	Asian	East Asian	Other Asian	Latin American 1	Latin American 2	South Asian	Other
rs1065852	Coding sequence variant	*CYP2D6*	G=0.788	G=0.782	G=0.864	G=0.948	G=0.861	G=0.421	G=0.427	G=0.410	G=0.832	G=0.876	G=0.800	G=0.785
	(Missense variant)		A=0.212	A=0.218	A=0.136	A=0.052	A=0.139	A=0.579	A=0.573	A=0.590	A=0.168	A=0.124	A=0.200	A=0.215
rs5030862	Coding sequence variant	*CYP2D6*	C=1.000	C=1.000	C=0.999	C=1.000	C=0.999	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000
	(Missense variant)		T=0.000	T=0.000	T=0.001	T=0.000	T=0.000	T=0.000	T=0.000	T=0.000	T=0.000	T=0.000	T=0.000	T=0.000
rs201377835	Splicing site variant	*CYP2D6*	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000
	(Splice acceptor variant)		G=0.000	G=0.000	G=0.000	G=0.000	G=0.000	G=0.000	G=0.000	G=0.000	G=0.000	G=0.000	G=0.000	G=0.000
rs28371706	Coding sequence variant	*CYP2D6*	G=0.987	G=0.998	G=0.911	G=0.851	G=0.913	G=1.000	G=1.000	G=1.000	G=0.969	G=1.000	G=1.000	G=0.988
	(Missense variant)		A=0.013, T=0.000	A=0.002, T=0.000	A=0.089, T=0.000	A=0.149, T=0.000	A=0.087, T=0.000	A=0.000, T=0.000	A=0.000, T=0.000	A=0.000, T=0.000	A=0.031, T=0.000	A=0.000, T=0.000	A=0.000, T=0.000	A=0.012, T=0.000
rs5030655	Coding sequence variant	*CYP2D6*	A=0.998	A=0.998	A=0.998	A=1.000	A=0.998	A=1.000	A=1.000	A=1.000	A=1.000	A=1.000	A=1.000	A=0.994
	(Frameshift variant)		delA=0.002	delA=0.002	delA=0.002	delA=0.000	delA=0.002	delA=0.000	delA=0.000	delA=0.000	delA=0.000	delA=0.000	delA=0.000	delA=0.006
rs5030865	Coding sequence variant	*CYP2D6*	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=0.982	C=0.991	C=0.960	C=1.000	C=1.000	C=1.000	C=1.000
	(Stop gained variant)		A=0.000, G=0.000, T=0.000	A=0.000, G=0.000, T=0.000	A=0.000, G=0.000, T=0.000	A=0.000, G=0.000, T=0.000	A=0.000, G=0.000, T=0.000	A=0.000, G=0.000, T=0.018	A=0.000, G=0.000, T=0.009	A=0.000, G=0.000, T=0.040	A=0.000, G=0.000, T=0.000	A=0.000, G=0.000, T=0.000	A=0.000, G=0.000, T=0.000	A=0.000, G=0.000, T=0.000
rs3892097	Splicing site variant	*CYP2D6*	C=0.818	C=0.809	C=0.907	C=0.975	C=0.905	C=0.995	C=0.994	C=1.000	C=0.860	C=0.889	C=0.790	C=0.814
	(Splice acceptor variant)		T=0.182	T=0.191	T=0.093	T=0.025	T=0.095	T=0.005	T=0.006	T=0.000	T=0.140	T=0.111	T=0.210	T=0.186
rs35742686	Coding sequence variant	*CYP2D6*	T=0.989	T=0.986	T=0.996	T=1.000	T=0.996	T=1.000	T=1.000	T=1.000	T=1.000	T=0.993	T=1.000	T=0.992
	(Frameshift variant)		delT=0.011	delT=0.014	delT=0.004	delT=0.000	delT=0.004	delT=0.000	delT=0.000	delT=0.000	delT=0.000	delT=0.007	delT=0.000	delT=0.008
rs16947	Coding sequence variant	*CYP2D6*	G=0.678	G=0.682	G=0.660	G=0.58	G=0.663	G=0.944	G=0.970	G=0.900	G=0.677	G=0.979	G=0.970	G=0.645
	(Missense variant)		A=0.322, T=0.000	A=0.318, T=0.000	A=0.340, T=0.000	A=0.42, T=0.00	A=0.337, T=0.000	A=0.056, T=0.000	A=0.030, T=0.000	A=0.100, T=0.000	A=0.323, T=0.000	A=0.021, T=0.000	A=0.030, T=0.000	A=0.355, T=0.000
rs5030867	Coding sequence variant	*CYP2D6*	T=0.999	T=0.999	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000	T=0.960	T=0.999
	(Missense variant)		G=0.001	G=0.001	G=0.000	G=0.000	G=0.000	G=0.000	G=0.000	G=0.000	G=0.000	G=0.000	G=0.040	G=0.001
rs28371725	Intronic variant	*CYP2D6*	C=0.909	C=0.896	C=0.961	C=0.975	C=0.961	C=0.957	C=0.954	C=0.970	C=0.881	C=0.962	C=0.886	C=0.896
			T=0.091	T=0.104	T=0.039	T=0.025	T=0.039	T=0.043	T=0.046	T=0.030	T=0.119	T=0.038	T=0.114	T=0.104

(Continue)

rs59421388	Coding sequence variant	*CYP2D6*	C=0.995	C=1.000	C=0.917	C=0.884	C=0.918	C=1.000	C=1.000	C=0.998	C=0.977	C=0.995	C=1.000	C=0.996
	(Missense variant)		T=0.005	T=0.000	T=0.083	T=0.116	T=0.083	T=0.000	T=0.000	T=0.002	T=0.023	T=0.005	T=0.000	T=0.004
rs1135840	Coding sequence variant	*CYP2D6*	C=0.426	C=0.433	C=0.380	C=0.450	C=0.377	C=0.280	C=0.282	C=0.280	C=0.425	C=0.577	C=0.400	C=0.413
	(Missense variant)		G=0.574, T=0.000	G=0.567, T=0.000	G=0.620, T=0.000	G=0.550, T=0.000	G=0.623, T=0.000	G=0.720, T=0.000	G=0.718, T=0.000	G=0.720, T=0.000	G=0.575, T=0.000	G=0.423, T=0.000	G=0.600, T=0.000	G=0.587, T=0.000
rs5030656	Coding sequence variant	*CYP2D6*	CTTCT=0.981	CTTCT=0.978	CTTCT=0.993	CTTCT=0.992	CTTCT=0.994	CTTCT=1.000	CTTCT=1.000	CTTCT=1.000	CTTCT=1.000	CTTCT=0.990	CTTCT=0.990	CTTCT=0.986
	(Missense variant)		CT=0.019	CT=0.022	CT=0.007	CT=0.008	CT=0.006	CT=0.000	CT=0.000	CT=0.000	CT=0.000	CT=0.010	CT=0.010	CT=0.014
rs774671100	Coding sequence variant	*CYP2D6*	A=1.000	A=1.000	A=1.000	A=1.000	A=1.000	A=1.000	A=1.000	A=1.000	A=1.000	A=1.000	A=1.000	A=1.000
	(Missense variant)		AA=0.000	AA=0.000	AA=0.000	AA=0.000	AA=0.000	AA=0.000	AA=0.000	AA=0.000	AA=0.000	AA=0.000	AA=0.000	AA=0.000
rs12248560	5' Upstream variant	*CYP2C19*	C=0.778	C=0.769	C=0.774	C=0.789	C=0.773	C=0.982	C=0.990	C=0.960	C=0.836	C=0.897	C=0.930	C=0.811
			T=0.222	T=0.231	T=0.226	T=0.211	T=0.227	T=0.018	T=0.010	T=0.040	T=0.164	T=0.103	T=0.070	T=0.189
	Initiator codon variant	*CYP2C19*	A=0.997	A=0.997	A=0.999	A=1.000	A=0.999	A=0.999	A=1.000	A=0.997	A=0.997	A=0.997	A=1.000	A=0.995
rs28399504	(Missense variant)		G=0.003	G=0.003	G=0.001	G=0.000	G=0.001	G=0.001	G=0.000	G=0.003	G=0.003	G=0.003	G=0.000	G=0.005
rs41291556	Coding sequence variant	*CYP2C19*	T=0.998	T=0.997	T=0.999	T=1.000	T=0.999	T=1.000	T=1.0000	T=1.000	T=1.000	T=0.999	T=1.000	T=0.998
	(Missense variant)		C=0.002	C=0.003	C=0.001	C=0.000	C=0.001	C=0.000	C=0.0000	C=0.000	C=0.000	C=0.001	C=0.000	C=0.002
rs72552267	Coding sequence variant	*CYP2C19*	G=1.000	G=1.000	G=1.000	G=1.000	G=1.000	G=1.000	G=1.000	G=1.000	G=1.000	G=0.998	G=1.000	G=1.000
	(Missense variant)		A=0.000	A=0.000	A=0.000	A=0.000	A=0.000	A=0.000	A=0.000	A=0.000	A=0.000	A=0.002	A=0.000	A=0.000
rs17884712	Coding sequence variant	*CYP2C19*	G=0.999	G=1.000	G=0.987	G=0.992	G=0.987	G=1.000	G=1.000	G=1.000	G=0.996	G=0.998	G=1.000	G=0.999
	(Missense variant)		A=0.001	A=0.000	A=0.013	A=0.008	A=0.013	A=0.000	A=0.000	A=0.000	A=0.004	A=0.002	A=0.000	A=0.001
rs4986893	Coding sequence variant	*CYP2C19*	G=0.992	G=0.994	G=1.000	G=0.997	G=1.000	G=0.919	G=0.921	G=0.914	G=0.999	G=1.000	G=0.997	G=0.987
	(Stop gained variant)		A=0.008	A=0.006	A=0.000	A=0.003	A=0.000	A=0.081	A=0.079	A=0.086	A=0.001	A=0.000	A=0.003	A=0.013
rs6413438	Coding sequence variant	*CYP2C19*	C=1.000	C=1.000	C=0.997	C=1.000	C=0.997	C=0.999	C=0.999	C=1.000	C=0.999	C=1.000	C=1.000	C=1.000
	(Missense variant)		T=0.000	T=0.000	T=0.003	T=0.000	T=0.003	T=0.001	T=0.001	T=0.000	T=0.001	T=0.000	T=0.000	T=0.000
rs4244285	Coding sequence variant	*CYP2C19*	G=0.850	G=0.853	G=0.825	G=0.816	G=0.825	G=0.708	G=0.720	G=0.660	G=0.838	G=0.891	G=0.633	G=0.848
	(Synonymous variant)		A=0.150, T=0.000	A=0.147, T=0.000	A=0.175, T=0.000	A=0.184, T=0.000	A=0.175, T=0.000	A=0.292, T=0.000	A=0.280, T=0.000	A=0.340, T=0.000	A=0.162, T=0.000	A=0.109, T=0.000	A=0.367, T=0.000	A=0.152, T=0.000
rs72558186	Splice donor variant	*CYP2C19*	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000	T=1.000
			C=0.000	C=0.0000	C=0.000	C=0.000	C=0.000	C=0.000	C=0.000	C=0.000	C=0.000	C=0.000	C=0.000	C=0.000
rs56337013	Coding sequence variant	*CYP2C19*	C=1.000	C=1.000	C=1.0000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000	C=1.000
	(Missense variant)		T=0.000	T=0.000	T=0.0000	T=0.000	T=0.000	T=0.000	T=0.000	T=0.000	T=0.000	T=0.000	T=0.000	T=0.000
rs489693	Intergenic variant	*MC4R*	C=0.700	C=0.694	C=0.612	C=0.602	C=0.612	C=0.791	C=0.783	C=0.811	C=0.654	C=0.825	C=0.629	C=0.710
			A=0.300, G=0.000, T=0.000	A=0.306, G=0.000, T=0.000	A=0.388, G=0.000, T=0.000	A=0.398, G=0.000, T=0.000	A=0.388, G=0.000, T=0.000	A=0.209, G=0.0000, T=0.0000	A=0.217, G=0.000, T=0.000	A=0.189, G=0.0000, T=0.0000	A=0.346, G=0.000, T=0.000	A=0.175, G=0.000, T=0.000	A=0.371, G=0.000, T=0.000	A=0.290, G=0.000, T=0.000
rs4713916	3' Downstream variant	*FKBP5*	A=0.304	A=0.311	A=0.141	A=0.190	A=0.140	A=0.410	A=0.330	A=0.800	A=0.600	A=0.545	A=0.580	A=0.255
			C=0.000, G=0.696, T=0.000	C=0.000, G=0.689, T=0.000	C=0.000, G=0.859, T=0.000	C=0.000, G=0.810, T=0.000	C=0.000, G=0.860, T=0.000	C=0.000, G=0.590, T=0.000	C=0.000, G=0.670, T=0.000	C=0.000, G=0.200, T=0.000	C=0.000, G=0.400, T=0.000	C=0.000, G=0.455, T=0.000	C=0.000, G=0.420, T=0.000	C=0.000, G=0.745, T=0.000
rs6295	5' Upstream variant	*HTR1A*	C=0.521	C=0.507	C=0.601	C=0.650	C=0.600	C=0.304	C=0.310	C=0.270	C=0.404	C=0.551	C=0.480	C=0.531
			A=0.000, G=0.479	A=0.000, G=0.493	A=0.0000, G=0.399	A=0.000, G=0.350	A=0.000, G=0.400	A=0.000, G=0.696	A=0.000, G=0.690	A=0.000, G=0.730	A=0.000, G=0.596	A=0.000, G=0.449	A=0.000, G=0.520	A=0.000, G=0.469
rs7997012	Intronic variant	*HTR2A*	A=0.396	A=0.427	A=0.072	A=0.000	A=0.074	A=0.245	A=0.228	A=0.360	A=0.244	A=0.337	A=0.385	A=0.319
			G=0.604	G=0.573	G=0.928	G=1.000	G=0.926	G=0.755	G=0.772	G=0.640	G=0.756	G=0.663	G=0.615	G=0.681

Table 3: Type of variant and allele frequency among biogeographic groups of all SNPs included in the PGx test (from: https://www.pharmgkb.org/).

Target variants located in *CYP2D6* and *CYP2C19* allow to identify 36 and 11 different haplotypes, respectively, and to potentially assign 703 and 78 different diplotypes. Frequencies among biogeographic groups of all identifiable haplotypes are shown in Table 4 and 5.

	Allele frequency in biogeographical groups								
CYP2D6 allele	African American/Afro-Caribbean	American	Central/South Asian	East Asian	European	Latino	Near Eastern	Oceanian	Sub-Saharan African
*1	0.2005736	0.5110660	0.2870746	0.2453141	0.2827814	0.3645527	0.2487631	0.7291202	0.0779216
*1x2	0.0080626	0.0286102	0.0056089	0.0034015	0.0082751	0.0150920	0.0314823	0.1188863	0.0111547
*2	0.1556877	0.2207781	0.2947621	0.1205126	0.1855969	0.2268406	0.1900492	0.0392258	0.1982548
*2x2	0.0187640	0.0060664	0.0095214	0.0045305	0.0084446	0.0118362	0.0331487	0.0000000	0.0173195
*3	0.0031705	0.0002383	0.0010578	0.0000584	0.0159059	0.0071794	0.0043491	0.0010405	0.0015354
*3x2	0.0000000	NA	NA	NA	0.0001000	0.0005000	NA	NA	NA
*4	0.0482296	0.1023906	0.0906299	0.0053711	0.1853820	0.1205136	0.1140594	0.0180000	0.0338422
*4x2	0.0265014	0.0010803	0.0008939	0.0000000	0.0065866	0.0040316	0.0032411	0.0000000	0.0152762
*5	0.0539319	0.0158736	0.0459194	0.0486342	0.0295285	0.0291579	0.0182356	0.0406163	0.0515362
*6	0.0028868	0.0024552	0.0000000	0.0001671	0.0111156	0.0051103	0.0054327	0.0000000	0.0000000
*6x2	0.0000678	0.0000000	NA	0.0000000	0.0000000	0.0001104	0.0000000	NA	NA
*7	0.0002719	0.0049643	0.0055033	0.0000789	0.0005324	0.0000000	0.0028612	0.0000000	0.0000000
*8	0.0000000	0.0010120	0.0000000	0.0000000	0.0002245	0.0000000	0.0000000	0.0000000	0.0000000
*9	0.0043548	0.0044353	0.0030100	0.0018527	0.0275758	0.0155005	0.0038029	0.0000000	0.0000000
*9x2	0.0000000	NA	NA	NA	0.0001000	0.0000000	NA	NA	NA
*10	0.0381630	0.0143721	0.0866698	0.4356336	0.0157463	0.0262916	0.0676936	0.0252314	0.0556769
*10x2	0.0011924	0.0000000	0.0000000	0.0060964	0.0001080	0.0016659	0.0022596	0.0000000	0.0000000
*11	0.0000000	0.0000000	NA	0.0000000	0.0001570	0.0000000	0.0000000	0.0000000	NA
*12	0.0008395	0.0170435	NA	0.0000000	0.0001535	NA	0.0000000	NA	0.0026000
*14	0.0000000	0.0000000	NA	0.0029519	0.0000000	0.0000000	NA	0.0000000	NA
*15	0.0000000	0.0024348	NA	0.0001002	0.0005137	0.0000000	0.0000000	0.0000000	0.0057000
*17	0.1688212	0.0047503	0.0006661	0.0000979	0.0039401	0.0232611	0.0309702	0.0010414	0.1928703
*17x2	0.0020345	0.0000000	NA	0.0000000	0.0000000	0.0088266	0.0000000	NA	NA
*29	0.0879710	0.0018643	0.0026858	0.0001085	0.0010577	0.0152478	0.0077560	0.0000000	0.1210733
*29x2	0.0010612	NA	NA	0.0000000	0.0000000	0.0001892	NA	NA	NA
*34	NA	NA	NA	0.0101913	0.0191317	0.0005000	NA	0.0018000	NA
*39	0.0268333	NA	0.0020000	0.0059056	0.0163306	0.0080000	0.0268444	0.0063142	0.0000000
*41	0.0372338	0.0232529	0.1229548	0.0226678	0.0924459	0.0510156	0.1535841	0.0075000	0.1146621

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*41x2	0.0006066	0.0000000	0.0009330	0.0002981	0.0003408	0.0000822	0.0043813	0.0000000	0.0023173
*64	NA	NA	NA	0.0000000	NA	NA	0.0000000	NA	0.0000000
*65	NA	NA	NA	0.0295290	NA	NA	0.0000000	NA	0.0000000
*69	NA	NA	NA	0.0117240	NA	NA	0.0000000	NA	NA
*70	NA	NA	NA	0.0000000	0.0000000	NA	0.0000000	NA	0.0000000
*91	NA	NA	NA	NA	NA	NA	NA	NA	NA
*109	NA	NA	NA	NA	NA	NA	NA	NA	NA
*114	NA	NA	NA	0.0007863	NA	NA	NA	NA	NA

Table 4: Frequencies of *CYP2D6* alleles identifiable by PGx algorithm for each biogeographical group; NA = not available (from: https://www.pharmgkb.org/).

	Allele frequency in biogeographical groups								
CYP2C19 allele	African American/Afro-Caribbean	American	Central/South Asian	East Asian	European	Latino	Near Eastern	Oceanian	Sub-Saharan African
*1	0.5467616	0.7921841	0.5436260	0.5955490	0.6251414	0.7165527	0.6722510	0.1870989	0.5515068
*2	0.1814949	0.1213653	0.2699160	0.2835226	0.1468570	0.1041875	0.1198471	0.6095202	0.1568425
*3	0.0027568	0.0003517	0.0156852	0.0724734	0.0016181	0.0008284	0.0164792	0.1463809	0.0026677
*4	0.0000000	0.0000187	0.0000000	0.0001774	0.0023597	0.0005203	0.0000000	NA	0.0000000
*5	0.0000000	0.0000000	0.0000000	0.0032252	0.0000288	0.0000000	0.0000000	NA	0.0000000
*6	0.0000000	NA	0.0000000	0.0005600	0.0003002	0.0000000	0.0000000	NA	0.0000000
*7	0.0000000	NA	0.0000000	0.0001138	0.0000000	0.0000000	NA	NA	0.0000000
*8	0.0011203	0.0000000	0.0000000	0.0000000	0.0033594	0.0012739	0.0000000	NA	0.0000000
*9	0.0143471	NA	NA	0.0001150	0.0006603	0.0008389	NA	NA	0.0269615
*10	0.0032672	NA	NA	0.0001047	0.0000000	0.0012398	0.0000000	NA	0.0000000
*17	0.2072274	0.0860802	0.1707728	0.0205413	0.2154386	0.1665585	0.1914227	0.0570000	0.1733359

Table 5: Frequencies of *CYP2C19* alleles identifiable by PGx algorithm for each biogeographical group; NA = not available (from: https://www.pharmgkb.org/).

PGx report generation

In compliance with the customized PGx guidelines, the algorithm classified all the pre-selected AD drugs in one of three categories on the basis of the patient's genotype. The following ADs have been included in the algorithm: agomelatine, amisulpride, amitriptyline, bupropion, citalopram, clomipramine, duloxetine, escitalopram, fluoxetine, fluvoxamine, imipramine, mianserine, mirtazapine, nortriptyline, paroxetine, reboxetine, sertraline, trazodone, trimipramine, and vortioxetine.

Classification criteria are summarised in Table 6 and Table 7.

Antidepressant	Gene	Metabolizer status	Main PGx evidence	Category of suggestion
Agomelatine, bupropion, duloxetine, fuoxetine, fluvoxamine, mianserine, mirtazapine, nortriptyline, reboxetine, trazodone, venlafaxine, vortioxetine	*CYP2C19*	All	No dosing recommendations on PharmGKB clinical guidelines	Green
Amitriptyline, citalopram, clomipramine, escitalopram, imipramine, paroxetine, sertraline, trimipramine	*CYP2C19*	NM	Normal metabolism. Initiate therapy with recommended starting dose.	Green
Amitriptyline, citalopram, clomipramine, escitalopram, imipramine, paroxetine, sertraline, trimipramine	*CYP2C19*	IM/LIM; RM/UM	IM/LIM: probable reduction in metabolism. Initiate therapy with recommended starting dose. RM/UM: probable increase in metabolism and reduction of clinic efficacy. Consider alternative drug not metabolized by CYP2C19.	Yellow
Amitriptyline, citalopram, clomipramine, escitalopram, imipramine, paroxetine, sertraline, trimipramine	*CYP2C19*	IM/LPM/PM; RM/UM	Reduced clinic efficacy and increased risk of side effects. Consider a reduction of starting dose or an alternative drug not metabolized by CYP2C19.	Red
Agomelatine, bupropion, citalopram, duloxetine, escitalopram, fluoxetine, mianserine, mirtazapine, reboxetine, sertraline, trazodone, vortioxetine	*CYP2D6*	All	No dosing recommendations on PharmGKB clinical guidelines	Green
Amitriptyline, clomipramine, fluvoxamine, impiramine, nortriptyline, trimipramine, venlafaxine	*CYP2D6*	NM	Normal metabolism. Initiate therapy with recommended starting dose.	Green
Fluvoxamine, paroxetine, trimipramine, venlafaxine	*CYP2D6*	IM; UM	IM: probable side effects.Initiate therapy with recommended starting dose. UM: probable reduction of clinic efficacy. Consider alternative drug not metabolized by CYP2D6.	Yellow
Amitriptyline, clomipramine, fluvoxamine, impiramine, nortriptyline, paroxeitine, trimipramine, venlafaxine	*CYP2D6*	IM/PM; UM	Reduced clinic efficacy and increased risk of side effects. Consider a reduction of starting dose or an alternative drug not metabolized by CYP2D6.	Red

Table 6: Summary of pharmacokinetics-related guidelines applied by the algorithm when ranking ADs in the final PGx report.

Antidepressant	Gene	Variant	Main PGx evidence	Category of suggestion
Citalopram, fluoxetine, mirtazapine, paroxetine, venlafaxine	*FKBP5*	rs4713916 (A/G, G/G)	Probable reduction of clinical efficacy	Yellow
Paroxetine	*HTR1A*	rs6295 (C/G, C/C)	Probable reduction of clinical efficacy	Yellow
Citalopram	*HTR2A*	rs7997012 (G/G)	Probable reduction of clinical efficacy	Yellow
Amisulpride	*MC4R*	rs489693 (A/A)	Probable increased risk of experiencing side effects, such as weight gain and hypertriglyceridemia	Yellow
Citalopram, escitalopram	*SLC6A4*	5-HTTLPR (LS/SS)	Probable reduction of clinical efficacy	Yellow
Citalopram	*SLC6A4*	5-HTTLPR (SS)	Reduction of clinical efficacy and increase of side effects risk	Red

Table 7: Summary of pharmacodynamics-related guidelines applied by the algorithm when ranking ADs in the final PGx report.

Report output

The final output of the algorithm is a report in PDF format in Italian (example in Figure 4). Additional information regarding starting dosage is made available to clinicians for each drug placed in the "Yellow" or "Red" categories. When information concerning gene-drug interactions are not available and the AD can't be allocated on the basis of the patient's genetic background, the drug is placed in the "Green" category, with a note regarding the lack of data.

Drug	Drug	Drug
Amitriptyline	Escitalopram	Agomelatine
Citalopram	Fluoxetine	Bupropion
Clomipramine	Mirtazapine	Duloxetine
Imipramine	Paroxetine	Fluvoxamine
Trimipramine	Sertraline	Mianserine
	Venlafaxine	Nortriptyline
		Reboxetine
		Trazodone
		Vortioxetine
		Amisulpride

Amitriptyline

[FC] Riduzione dell'efficacia clinica e maggiore probabilità di effetti collaterali. Ridurre del 50% la dose iniziale rispetto a quella raccomandata.[1]

Citalopram

[FC] Riduzione dell'efficacia clinica. Si consiglia la scelta di una molecola alternativa tenendo anche in considerazione alcune caratteristiche del paziente (es età, funzionamento renale ed epatico).[2]

[FD] Riduzione dell'efficacia clinica e aumento del rischio di effetti collaterali [3]

Clomipramine

[FC] Riduzione dell'efficacia clinica e maggiore probabilità di effetti collaterali. Si consiglia una molecola alternativa.[4]

Imipramine

[FC] Riduzione dell'efficacia clinica e maggiore probabilità di effetti collaterali. Si consiglia una molecola alternativa.[4]

Trimipramine

[FC] Riduzione dell'efficacia clinica e maggiore probabilità di effetti collaterali. Si consiglia una molecola alternativa.[4]

Escitalopram

[FC] Riduzione dell'efficacia clinica. Si consiglia la scelta di una molecola alternativa tenendo anche in considerazione alcune caratteristiche del paziente (es età, funzionamento renale ed epatico).[2]

[FD] Possibile riduzione dell'efficacia clinica [5]

Fluoxetine

[FC] Nessun dato disponibile.[6]

[FD] Possibile riduzione dell'efficacia clinica [5]

Mirtazapine

[FC] Normale metabolizzatore. Iniziare la terapia alla dose terapeutica raccomandata.[7]

[FD] Possibile riduzione dell'efficacia clinica [5]

Paroxetine

[FC] Normale metabolizzatore. Iniziare la terapia alla dose terapeutica raccomandata.[7]

[FD] Possibile riduzione dell'efficacia clinica[5]

Sertraline

[FC] Probabile incremento del metabolismo, non clinicamente rilevante. Iniziare la terapia alla dose terapeutica raccomandata. Si consiglia la scelta di una molecola alternativa tenendo anche in considerazione alcune caratteristiche del paziente (es età, funzionamento renale ed epatico).[8]

Venlafaxine

[FC] Normale metabolizzatore. Iniziare la terapia alla dose terapeutica raccomandata.[7]

[FD] Possibile riduzione dell'efficacia clinica[5]

Agomelatine

[FC] Nessun dato disponibile.[6]

Bupropion

[FC] Nessun dato disponibile.[6]

Duloxetine

[FC] Nessun dato disponibile.[6]

Fluvoxamine

[FC] Normale metabolizzatore. Iniziare la terapia alla dose terapeutica raccomandata.[7]

Mianserine

[FC] Nessun dato disponibile.[6]

Nortriptyline

[FC] Normale metabolizzatore. Iniziare la terapia alla dose terapeutica raccomandata.[7]

Reboxetine

[FC] Nessun dato disponibile.[6]

Trazodone

[FC] Nessun dato disponibile.[6]

Vortioxetine

[FC] Nessun dato disponibile.[6]

Amisulpride

Nessun dato disponibile[6]

Figure 4: Example of traffic-light style report. [1]Reduction of clinical efficacy and increased probability of side effects. Consider a 50% reduction of the recommended starting dose; [2]Reduction of clinical efficacy. Consider an alternative

molecule, also taking into account the patient's characteristics (e.g., age, renal and hepatic functioning); [3]Reduction of clinical efficacy and increased probability of side effects; [4]Reduction of clinical efficacy and increased probability of side effects. Consider an alternative drug; [5]Possible reduction of clinical efficacy; [6]No data available; [7]Normal metabolizer. Initiate therapy with the recommended starting dose; [8]Probable increase of metabolism, not clinically relevant. Initiate therapy with the recommended dose. Consider an alternative drug, also taking into account the patient's clinical features (e.g., age, kidney and hepatic functioning).

The PGx test herein described is to date under evaluation in a clinical trial, as described in Minelli et al. 2021. The aim of the study is to investigate whether a non-commercial PGx test could improve AD outcomes in a cohort of 300 MDD Italian patients referred to psychiatric service to receive a new AD therapy because of the failure of their current treatment or the onset of side effects. Recruitment of patients officially started on February 1st, 2020 and it was expected to be concluded by the end of this PhD project but, because of the COVID-19 pandemic, patients' recruitment has been interrupted for about eight months (February-September 2020) and several difficulties in recruiting have also occurred after partial easing of restrictions. To date, approximately 170 patients have been recruited and, consequently, final data were not available for the analysis at the time of writing.

5. Retrospective evaluation of PGx test usefulness in guiding clinical decision-making

5.1. Aim of the work

The aim of this study was to assess the ability of the PGx algorithm described in Chapter 4 in predicting response of MDD patients to AD treatments. We tested the validity of our PGx test by performing a retrospective analysis on a cohort of MDD patients whose information on AD treatment outcomes was already available. The purpose of this retrospective analysis was to assess whether our PGx algorithm could have been able to predict AD response in these MDD patients and, consequently, to evaluate whether the test could have been useful to avoid multiple trials before finding an efficient treatment.

5.2. Material and methods

Study participants

A total of 157 MDD patients with Italian ancestry were selected among a cohort of individuals previously recruited for other studies on psychiatric disorders. The eligibility criteria for our study consisted in the diagnosis of moderate to severe MDD according to the DSM-IV criteria.

Depending on the efficacy of the pharmacological trial, each drug was classified in a binary fashion, i.e. "failed treatment" or "successful treatment". In particular, when the patient did not achieve a satisfactory outcome and needed an augmentation or a switch to another AD, treatment was defined as "failed". Contrarily, when an improvement in MDD symptoms was observed, and the patient didn't need a change in management, the treatment was defined as "successful". During the first AD trial, seventy-four patients were successfully treated, whereas 83 failed the first treatment and needed a switching or an augmentation with another AD.

All patients were characterized based on the following clinical and demographic features: gender, age at MDD onset, age at first AD trial, smoking, family history for psychiatric disease (depression, bipolar disease, anxiety disorder, schizophrenia, other psychiatric disorder), alcohol abuse, and suicide. Groups of success did not significantly differ for any of these variables (Table 8).

	Successful treatment (N=83)	Failed treatment (N=74)	Overall (N=157)	p-value
Gender				
Male	21 (25.3%)	28 (37.8%)	49 (31.2%)	0.12
Female	62 (74.7%)	46 (62.2%)	108 (68.8%)	
Age at the first AD treatment (Years)				
Mean (SD)	40.8 (16.1)	40.6 (13.8)	40.7 (15.0)	0.96
Median [Min, Max]	40.0 [5.00, 83.0]	40.5 [17.0, 76.0]	40.0 [5.00, 83.0]	
Smoking				
No	61 (73.5%)	47 (63.5%)	108 (68.8%)	0.23
Yes	22 (26.5%)	27 (36.5%)	49 (31.2%)	
Alcohol abuse				
No	81 (97.6%)	71 (95.9%)	152 (96.8%)	0.67
Yes	2 (2.4%)	3 (4.1%)	5 (3.2%)	
Onset age (Years)				
Mean (SD)	39.3 (16.1)	37.8 (14.8)	38.6 (15.5)	0.58
Median [Min, Max]	38.0 [11.0, 83.0]	38.0 [14.0, 76.0]	38.0 [11.0, 83.0]	
Familiarity with psychiatric diseases				
No	30 (36.1%)	21 (28.4%)	51 (32.5%)	0.32
Depression	46 (55.4%)	39 (52.7%)	85 (54.1%)	
Bipolar Disease	3 (3.6%)	5 (6.8%)	8 (5.1%)	
Anxiety Disorder	0 (0%)	2 (2.7%)	2 (1.3%)	
Schizophrenia	4 (4.8%)	7 (9.5%)	11 (7.0%)	
Other	0 (0%)	0 (0%)	0 (0%)	
Suicide				
No	76 (91.6%)	67 (90.5%)	143 (91.1%)	1
Yes	7 (8.4%)	7 (9.5%)	14 (8.9%)	

Table 8: Clinical and demographic features of patients with successful or failed treatment at first AD trial.

Sample processing and DNA extraction

Genomic DNA (gDNA) was extracted from blood samples using the QIAmp DNA blood Maxi kit (Qiagen, Hilden, Germany) and evaluated for quality and concentration with NanoDrop Spectrophotometer 2000 (Thermo Fisher Scientific, Waltham, USA). gDNA samples were then stored at -20° until use.

Genotyping

The genotyping of different gene variants (SNPs, CNV, insertion/deletion) was conducted with specific methodologies.

Genotyping of a total number of 29 SNPs (10 in *CYP2C19*, 15 *CYP2D6*, and four in *FKBP5, MC4R, HTR1A,* and *HTR2A*) was carried out on 125 ng of gDNA using a customized TaqMan™ OpenArray™

Genotyping plate on the QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems™, Foster City, California, USA) according to the manufacturer's protocol. Each sample was genotyped twice in order to reduce error rate and avoid undetermined/undefined results. After each run, raw data were manually inspected and analysed with the Thermo Fisher Cloud Genotyping application. Genotyping results were exported for haplotypes assignment.

Copy number of *CYP2D6* were evaluated in 10 ng of gDNA using the TaqMan® Copy Number Assay mix specific for exon 9 (Assay ID: Hs00010001_cn) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems™) according to the manufacturer's protocol. Samples' genotyping was conducted in triplicate and RNAse P was used as reference assay for all samples. For each target, cycle threshold (CT) was set at 0.2 to allow comparison of different runs. Then, data were exported and analysed with the CopyCaller® software (Applied Biosystems™) using the following setting: no calibrator sample, most frequent sample copy number = 2. Only CNV results with confidence ≥ 95% and Z-score between 1.75 and 2.65 were considered. Samples with low quality results were repeated.

SNPs and CNV genotyping results were then analysed through the AlleleTyper™ software to determine *CYP2C19* and *CYP2D6* diplotypes. AlleleTyper™ translated SNPs and CNV information to the corresponding haplotype/star allele (according to a gene-specific translation table available on PharmGKB) and provided all the possible diplotype combinations for each patient. When more than one diplotype was assigned, the most frequent in Europeans was selected. Phenotype prediction was performed for each patient considering the PharmGKB gene-specific diplotype-phenotype table, which maps each diplotype to a possible phenotype considering the haplotypes AS. Basing on the diplotypes identifiable using our genotyping panel, patients could be assigned to 7 metabolizer statuses for *CYP2C19* (i.e., ultrarapid - UM, rapid - RM, intermediate - IM, poor - PM, normal - NM, likely intermediate - LIM, and likely poor metabolizer -LPM) and 4 for *CYP2D6* (i.e., UM, NM, IM, and PM).

5-HTTLPR genotyping

The 5-HTTLPR VNTR was amplified by Polymerase Chan Reaction (PCR) using 50 ng of gDNA with the KAPA HiFi HotStart PCR Kit (Roche Diagnostics, Basel, Switzerland) and customized primers (forward: 5' – ATG CCA GCA CCT AAC CCC TAA TGT – 3'; reverse: 5' – GGA CCG CAA GGT GGG CGG GA – 3'). PCR was conducted on a SimpliAmp™ Thermal Cycler (Applied Biosystems™) with the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of 98°C denaturation for 20 sec,

annealing and extension at 72°C for 30 sec, and final extension of 1 min at 72°C. For each sample, 15 µl of the amplified DNA was analysed with a 2% agarose TBE gel to discriminate the long/short (L/S) alleles.

Report generation and drugs allocation

PDF PGx reports for each patient were generated as described in Chapter 4.

Statistical analyses

Statistical analyses were performed using the R Studio software (version 4.1.3). Clinical and demographic differences between patients with first medication in "successful treatment" and "failed treatment" were assessed using Fisher's Exact test for categorical variables. Continuous variables were tested for normality using the Shapiro-Wilk's test and differences between the two groups of response were assessed using the non-parametric Kruskal-Wallis' test.

Fisher's Exact tests or Pearson's Chi-Square tests were performed to identify any difference in the number of drugs allocated in the three PGx classes of suggestion between the two groups of response. Fisher's exact test was applied when at least one group of comparison has $n < 5$, otherwise Pearson's Chi-Square test was conducted.

Two-tailed post-hoc row-wise tests were performed to identify whether any PGx recommendation class significantly differed in terms of number of drugs between the two groups of response (*rstatix* R package). Correction for multiple tests using the Bonferroni's method was performed in post-hoc analyses. The odds ratio was calculated using binomial logistic regression. P-value < 0.05 and $p_{adj} < 0.05/n_{tests}$ were considered as statistically significant for Fisher's Exact test and post-hoc analysis, respectively.

5.3. Results

After the first AD trial, patients who did not respond adequately to the therapy underwent a second AD treatment attempt. Among these, 55 (66.27%) were successfully treated, whereas the others needed a third AD. A total of 9 patients (47%) failed also the third treatment attempt and needed a fourth one, eventually successful for 6 of them (Figure 5).

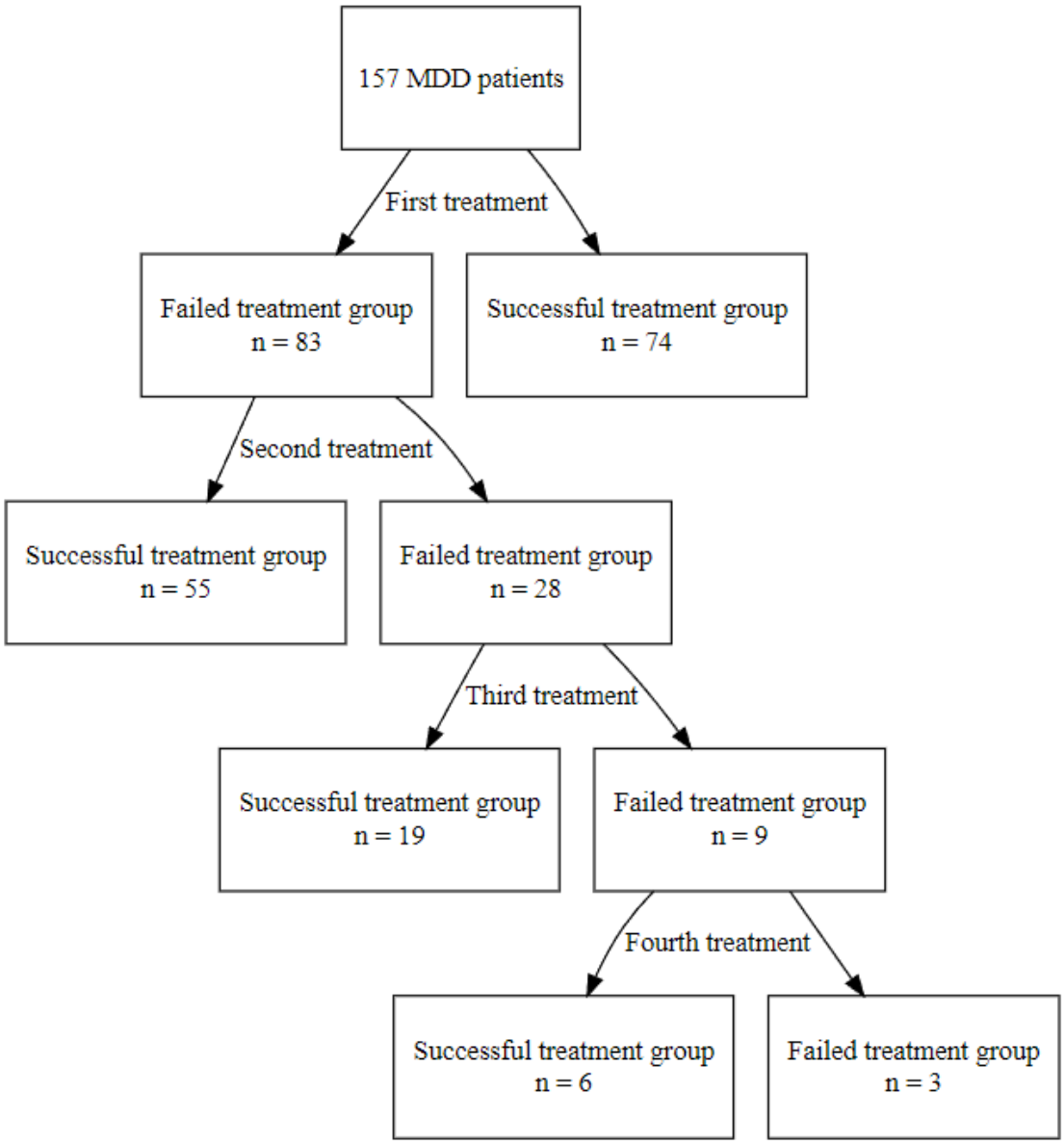

Figure 5: Flowchart of AD treatments outcome.

Retrospective analysis of drug administration and consistence with PGx suggestions

A total of 277 pharmacological treatments with different AD molecules have been administered in the whole MDD cohort throughout the 4 trial attempts, which could include a switching to another medication or a treatment augmentation. Among these, 142 (51.26%) were placed in the "Green" recommendation category for the specific patient, 91 were in the "Yellow" category (32.85%), whereas 44 (15.88%) were in the "Red" one (Table 9).

	First AD trial				Second AD trial				Third AD trial			
	Successful treatment	%	Failed treatment	%	Successful treatment	%	Failed treatment	%	Successful treatment	%	Failed treatment	%
Green	27	36.49	41	49.40	36	65.45	14	50.00	11	57.89	8	88.89
Yellow	31	41.89	26	31.33	17	30.91	6	21.43	6	31.58	1	11.11
Red	16	21.62	16	19.28	2	3.64	8	28.57	2	10.53	0	0.00
Total	74	100.00	83	100.00	55	100.00	28	100.00	19	100.00	9	100.00

Fourth AD trial				Total AD trials					
Successful treatment	%	Failed treatment	%	Successful treatment	%	Failed treatment	%	Total AD	%
4	66.67	1	33.33	78	50.65	64	52.03	142	51.26
2	33.33	2	66.67	56	36.36	35	28.46	91	32.85
0	0.00	0	0.00	20	12.99	24	19.51	44	15.88
6	100.00	3	100.00	154	100.00	123	100.00	277	100.00

Table 9. Classification of AD administered in each trial on the basis of PGx report for each group of response ("successful treatment" and "failed treatment").

No difference was observed in AD drugs distribution among PGx categories considering total AD trials. However, when single trial attempts were evaluated, a significant difference was observed in drug distribution in the second one ($p = 0.007$). In particular, post-hoc analysis revealed a higher number of drugs in the "Red" category in the failed treatment group compared to the successful treatment group (28.6% vs 3.64%, $p_{adj} = 0.006$, $n_{tests} = 3$), with treatments placed in the "Red" category having 10.1-fold higher odds of failure compared to drugs in other categories (OR: 10.1, 95% CI [2.07, 54.2]) (Figure 6).

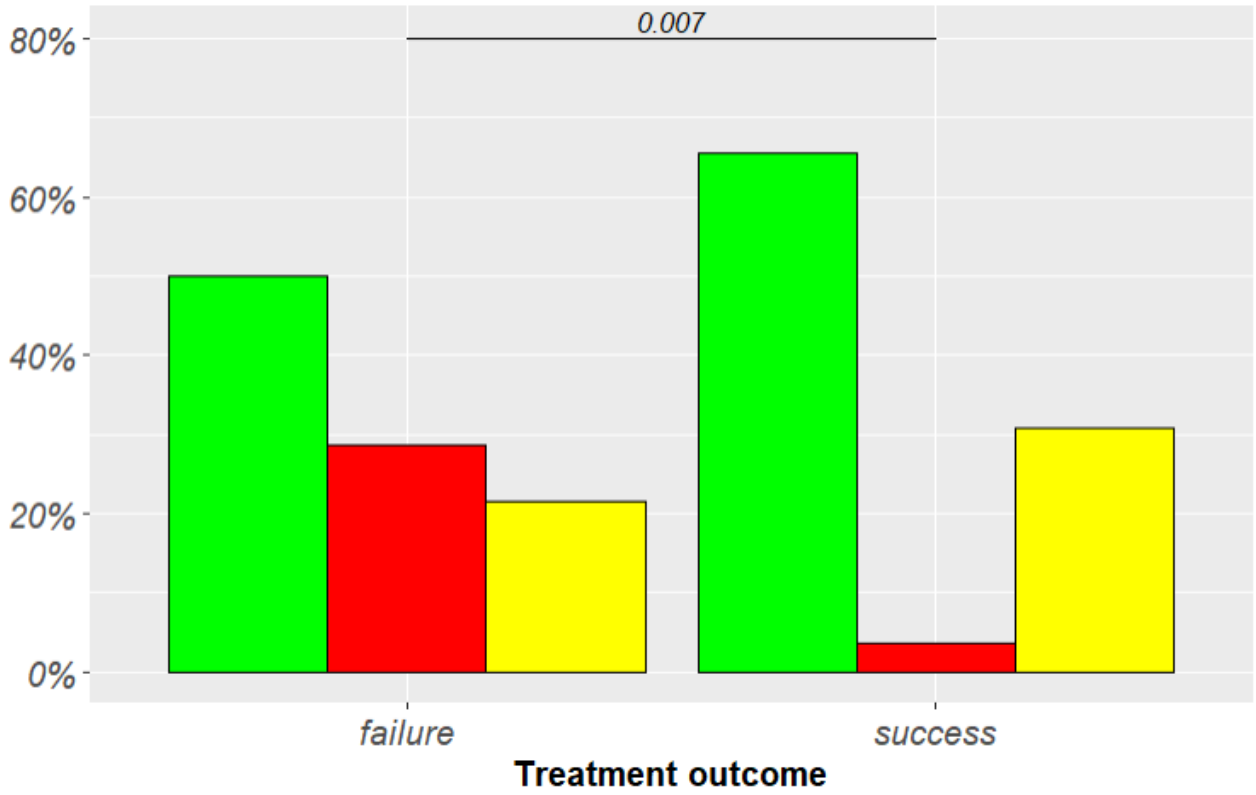

Figure 6: Bar-plots showing the classification of ADs on the basis of PGx report in the second treatment attempt.

Side effect prediction

A total of 24 AD treatments (on the 277 performed) induced the onset of side effects in the MDD cohort (8.66%) (Table 10). No difference was found in the distribution of AD treatments among PGx recommendation categories between patients developing side effects and those not developing them, both in terms of total AD trials and within each trial.

	First AD trial				Second AD trial				Third AD trial			
	Side effects	%	No side effects	%	Side effects	%	No side effects	%	Side effects	%	No side effects	%
Green	12	70.59	56	40.00	5	83.33	45	58.44	0	NaN	19	67.86
Yellow	4	23.53	53	37.86	1	16.67	22	28.57	0	NaN	7	25.00
Red	1	5.88	31	22.14	0	0.00	10	12.99	0	NaN	2	7.14
Total	17	100.00	140	100.00	6	100.00	77	100.00	0	NaN	28	100.00

(Continue)

Fourth AD trial				Total AD trials			
Side effects	%	No side effects	%	Side effects	%	No side effects	%
1	100	4	50	18	75.00	124	49.01
0	0	4	50	5	20.83	86	33.99
0	0	0	0	1	4.17	43	17.00
1	100	8	100	24	100.00	253	100.00

Table 10. Classification of ADs administered in each trial on the basis of PGx report for each group of side effects ("side effects" and "no side effects").

6. Candidate gene study to identify potential associations between AD PGx genes and Treatment Resistant Depression

6.1. Aim of the study

The aim of this part of the study was to contribute to clarify the genetic background of TRD by analysing those genes that have been previously associated with response to AD treatments. To this purpose, four members of CYP family and *SLC6A4* were selected and evaluated in two cohorts of MDD patients with opposite response-related phenotypes: responders to the first AD trial ("responder" group) and non-responders to multiple pharmacological treatments ("TRD" group).

6.2. Material and methods

Study participants

A total of 184 MDD patients of Italian ancestry (84 TRD and 100 responders) were included in the study. Patients were recruited at the Psychiatric Hospital "Villa Santa Chiara", Verona, Italy and at the IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli, Brescia, Italy. Diagnosis of moderate to severe MDD according to the DSM-IV was confirmed for all patients using the Structured Clinical Interview for DSM-IV Axis-I Disorder (SCID-I).

Patients were defined as suffering from TRD when they failed to respond to two or more adequate treatments with two or more different AD classes and to an adequate trial with TCAs, as described in Stage III of Thase and Rush Staging Method (Thase and Rush 1997). The group of responders included MDD patients achieving response or remission, in term of symptomatology reduction, after the first adequate AD trial.

Exclusion criteria were: mental retardation or cognitive disorder; history of schizophrenic, schizoaffective, or bipolar disorder; primary diagnosis of personality disorder, substance/alcohol abuse or dependency, obsessive compulsive disorder, post-traumatic stress disorder; comorbidity with eating disorders.

For all patients included in the study the following clinical and demographic data were collected: gender, age, education, age of MDD onset, Montgomery-Asberg depression rating scale (MADRS) score, recurrence, presence of psychotic symptoms or comorbidities (personality disorder, anxiety disorder, and general medical conditions), smoking status, and body mass index (BMI).

Sample processing and DNA extraction

Whole blood samples were collected through EDTA blood vacutainer tubes and gDNA was extracted with the QIAmp DNA Bood Maxi kit (Qiagen). gDNA samples were evaluated with NanoDrop Spectrophotometer 2000 (Thermo Fisher Scientific) for quality and concentration and were stored at -20° until use.

Attribution of cytochromes metabolizer phenotype

Four members of CYP family were chosen as pharmacokinetics targets: *CYP2B6*, *CYP2C9*, *CYP2C19*, and *CYP2D6.*

Genotyping of 4 SNPs in *CYP2B6*, 2 in *CYP2C9*, 10 in *CYP2C19* and 15 in *CYP2D6*, CNV evaluation of *CYP2D6,* and metabolizer phenotype prediction have been conducted as described in the "Materials and Methods" section of Chapter 5.

Basing on the diplotypes identifiable through our genotyping panel, each patient was assigned to one of five metabolizer statuses for *CYP2B6* (i.e., UM, RM, NM, IM, and PM), one of three for *CYP2C9* (i.e., NM, IM, and PM), one of seven for *CYP2C19* (i.e., UM, RM, IM, PM, NM, LIM, and LPM), and one of four for *CYP2D6* (i.e., UM, NM, IM, and PM).

5-HTTLPR and rs25531 genotyping

5-HTTLPR genotyping has been performed as described in the "Materials and Methods" section of Chapter 5.

The rs25531 SNP genotype was determined through digestion of 15 μl of PCR product with the restriction enzyme *MspI* and migration on a 3.5% agarose TBE gel.

Statistical analyses

Statistical analyses were conducted using the R Studio software (version 4.1.3). Differences in demographic and clinical characteristics between the groups of responders and TRD patients were assessed by two-tailed Student's t or Kruskal-Wallis' tests for continuous variables and Fisher's Exact test for categorical variables. Normality in data distribution was assessed using the Shapiro-Wilk's test. Differences in frequencies of cytochromes' metabolizer statuses and *SLC6A4* genotypes between TRD and responders have been verified by Pearson's Chi-Square test ($n > 5$) or Fisher's Exact test ($n < 5$). Logistic regression models were built to predict the probability of TRD for each

CYP metabolizer status and according to the presence of genetic variants in *SLC6A4.* Analyses were conducted using a binomial generalised linear model (*glm* R function) and corrected for confounding variables. A p-value < 0.05 was considered statistically significant.

6.3. Results

Clinical and demographic assessment

TRD and responder groups significantly differed for age, BMI, age of MDD onset, recurrence of depressive episodes, MADRS score, presence of psychotic symptoms and comorbidities including personality disorder, anxiety disorder, and general medical conditions (Table 11). These variables were included as covariates in the logistic regression analysis.

	TRD (N=84)	Responder (N=100)	Overall (N=184)	p-value
Gender				
Male	22 (26.2%)	32 (32.0%)	54 (29.3%)	0.39
Female	62 (73.8%)	68 (68.0%)	130 (70.7%)	
Age (Years)				
Mean (SD)	47.2 (15.3)	55.6 (11.4)	51.8 (13.9)	<0.05
Median [Min, Max]	45.0 [20.0, 87.0]	55.0 [30.0, 82.0]	51.0 [20.0, 87.0]	
BMI				
Mean (SD)	23.3 (4.81)	25.8 (4.58)	24.8 (4.82)	<0.05
Median [Min, Max]	22.6 [14.6, 40.4]	25.7 [17.7, 39.5]	24.7 [14.6, 40.4]	
Missing	23 (27.4%)	8 (8.0%)	31 (16.8%)	
Smoking				
No	41 (48.8%)	56 (56.0%)	97 (52.7%)	0.73
Yes	31 (36.9%)	38 (38.0%)	69 (37.5%)	
Missing	12 (14.3%)	6 (6.0%)	18 (9.8%)	
Education (Years)				
Mean (SD)	10.9 (3.57)	10.6 (3.96)	10.7 (3.78)	0.49
Median [Min, Max]	13.0 [5.00, 18.0]	11.0 [5.00, 19.0]	11.0 [5.00, 19.0]	
Onset age (Years)				
Mean (SD)	40.6 (13.8)	32.8 (13.0)	36.4 (13.9)	<0.05
Median [Min, Max]	38.5 [15.0, 86.0]	32.0 [14.0, 66.0]	34.0 [14.0, 86.0]	
Recurrence				
No	41 (48.8%)	8 (8.0%)	49 (26.6%)	<0.05
Yes	43 (51.2%)	92 (92.0%)	135 (73.4%)	
MADRS				
Mean (SD)	27.4 (5.17)	32.1 (6.84)	30.0 (6.56)	<0.05
Median [Min, Max]	26.0 [17.0, 45.0]	32.0 [18.0, 47.0]	29.0 [17.0, 47.0]	
Psychotic symptoms				
No	78 (92.9%)	68 (68.0%)	146 (79.3%)	<0.05
Yes	6 (7.1%)	32 (32.0%)	38 (20.7%)	
Comorbidity: personality disorder				
No	74 (88.1%)	51 (51.0%)	125 (67.9%)	<0.05
Yes	10 (11.9%)	49 (49.0%)	59 (32.1%)	
Comorbidity: anxiety disorder				
No	61 (72.6%)	50 (50.0%)	111 (60.3%)	<0.05
Yes	23 (27.4%)	50 (50.0%)	73 (39.7%)	
Comorbidity: general medical condition				
No	66 (78.6%)	59 (59.0%)	125 (67.9%)	<0.05
Yes	18 (21.4%)	41 (41.0%)	59 (32.1%)	

Table 11: Clinical and demographic features of TRD and responder groups.

Frequencies of metabolizer phenotypes and metabolic activity groups

Distribution of metabolizer phenotypes classes in TRD and responder groups are reported in Table 12. For one patient, assignment of a specific *CYP2B6* metabolizer status was not possible due to the presence of a diplotype sequence not included in the diplotype-phenotype translation table provided by PharmGKB. No difference was found in metabolizer phenotype distribution between the two groups for each cytochrome.

	TRD (N=84)	Responder (N=100)	Overall (N=184)	p-value
CYP2B6				
IM	34 (40.5%)	47 (47.0%)	81 (44.0%)	0.63
NM	37 (44.0%)	39 (39.0%)	76 (41.3%)	
PM	11 (13.1%)	9 (9.0%)	20 (10.9%)	
RM	2 (2.4%)	4 (4.0%)	6 (3.3%)	
Missing	0 (0%)	1 (1.0%)	1 (0.5%)	
CYP2C9				
IM	26 (31.0%)	31 (31.0%)	57 (31.0%)	0.89
NM	54 (64.3%)	66 (66.0%)	120 (65.2%)	
PM	4 (4.8%)	3 (3.0%)	7 (3.8%)	
CYP2C19				
IM	26 (31.0%)	20 (20.0%)	46 (25.0%)	0.28
NM	24 (28.6%)	41 (41.0%)	65 (35.3%)	
PM	4 (4.8%)	3 (3.0%)	7 (3.8%)	
RM	28 (33.3%)	32 (32.0%)	60 (32.6%)	
UM	2 (2.4%)	4 (4.0%)	6 (3.3%)	
CYP2D6				
IM	38 (45.2%)	36 (36.0%)	74 (40.2%)	0.49
NM	39 (46.4%)	56 (56.0%)	95 (51.6%)	
PM	3 (3.6%)	5 (5.0%)	8 (4.3%)	
UM	4 (4.8%)	3 (3.0%)	7 (3.8%)	

Table 12: Frequencies of cytochromes metabolizer statuses of TRD and responder groups.

Also, metabolic activity groups were considered for each cytochrome (Table 13). No difference was found in metabolic activity groups distribution between TRD and responders for each cytochrome.

	TRD (N=84)	Responder (N=100)	Overall (N=184)	p-value
CYP2B6 metabolic activity				
Increased (RM)	2 (2.4%)	4 (4.0%)	6 (3.3%)	0.71
Normal (NM)	37 (44.0%)	39 (39.0%)	76 (41.3%)	
Reduced (PM+IM)	45 (53.6%)	56 (56.0%)	101 (54.9%)	
Missing	0 (0%)	1 (1.0%)	1 (0.5%)	
CYP2C9 metabolic activity				
Normal (NM)	54 (64.3%)	66 (66.0%)	120 (65.2%)	0.81
Reduced (PM+IM)	30 (35.7%)	34 (34.0%)	64 (34.8%)	
CYP2C19 metabolic activity				
Increased (RM+UM)	30 (35.7%)	36 (36.0%)	66 (35.9%)	0.1
Normal (NM)	24 (28.6%)	41 (41.0%)	65 (35.3%)	
Reduced (PM+IM)	30 (35.7%)	23 (23.0%)	53 (28.8%)	
CYP2D6 metabolic activity				
Increased (UM)	4 (4.8%)	3 (3.0%)	7 (3.8%)	0.41
Normal (NM)	39 (46.4%)	56 (56.0%)	95 (51.6%)	
Reduced (PM+IM)	41 (48.8%)	41 (41.0%)	82 (44.6%)	

Table 13: Summary of the grouping by enzymatic activity for each member of CYP family included in the study.

Association between cytochrome metabolizer status and response phenotype

For each cytochrome included in the study (*CYP2B6*, *CYP2C9*, *CYP2C19*, and *CYP2D6*), association between metabolizer statuses and response phenotype (TRD or responder) was evaluated through logistic regressions as follows:

1. Considering the risk of TRD in patients with a non-normal metabolizer status (PM, IM, RM, or UM) compared to that of NM patients.
2. Considering the risk of TRD in patients with a non-functional enzyme (PM) compared to any other enzymatic activity (IM + NM + RM + UM).
3. Considering the risk of TRD in patients with a reduced (PM + IM) or an increased (RM + UM) enzymatic activity compared to normal phenotype (NM).

The responder group and the NM phenotype were considered the reference in all comparisons.

When the impact of enzymatic activity level on response phenotype was considered individually for each metabolizer phenotype (1st comparison), a significantly increased probability of TRD was observed in *CYP2C19* UM patients, compared to NM ($p = 0.01$, OR: 41.26, 95% CI [2.13, 800]) (Figure 7).

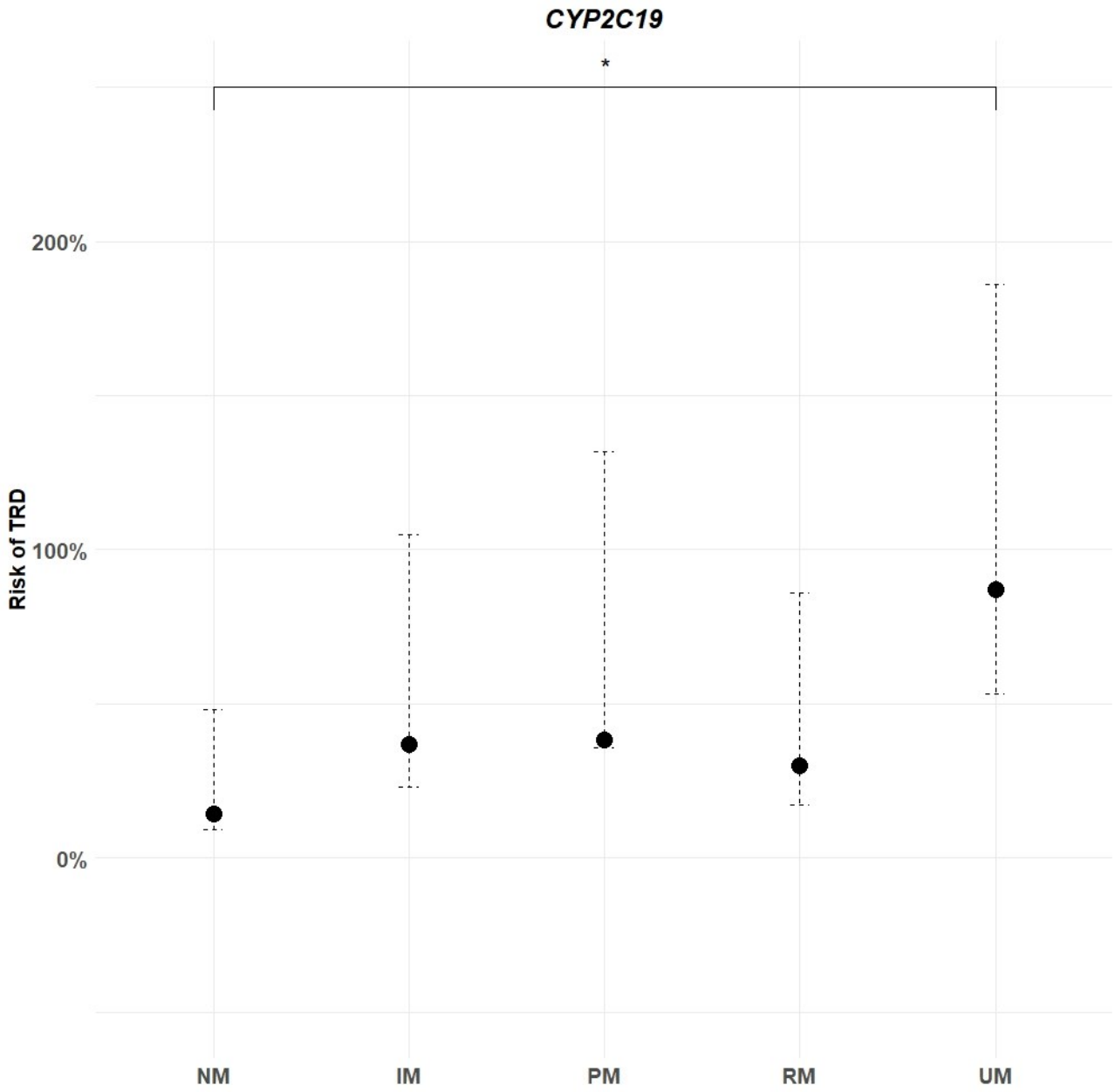

Figure 7: Main effect plot showing the predicted probability of TRD according to the *CYP2C19* metabolizer status. Dashed lines represent 95% CIs.

Contrarily, patients having a low CYP2C9 enzymatic activity (PM + IM) showed a trend for a reduced probability of TRD compared to patients with a normal metabolism (p = 0.09, OR: 0.35, 95% CI [0.10, 1.19]) (Figure 8).

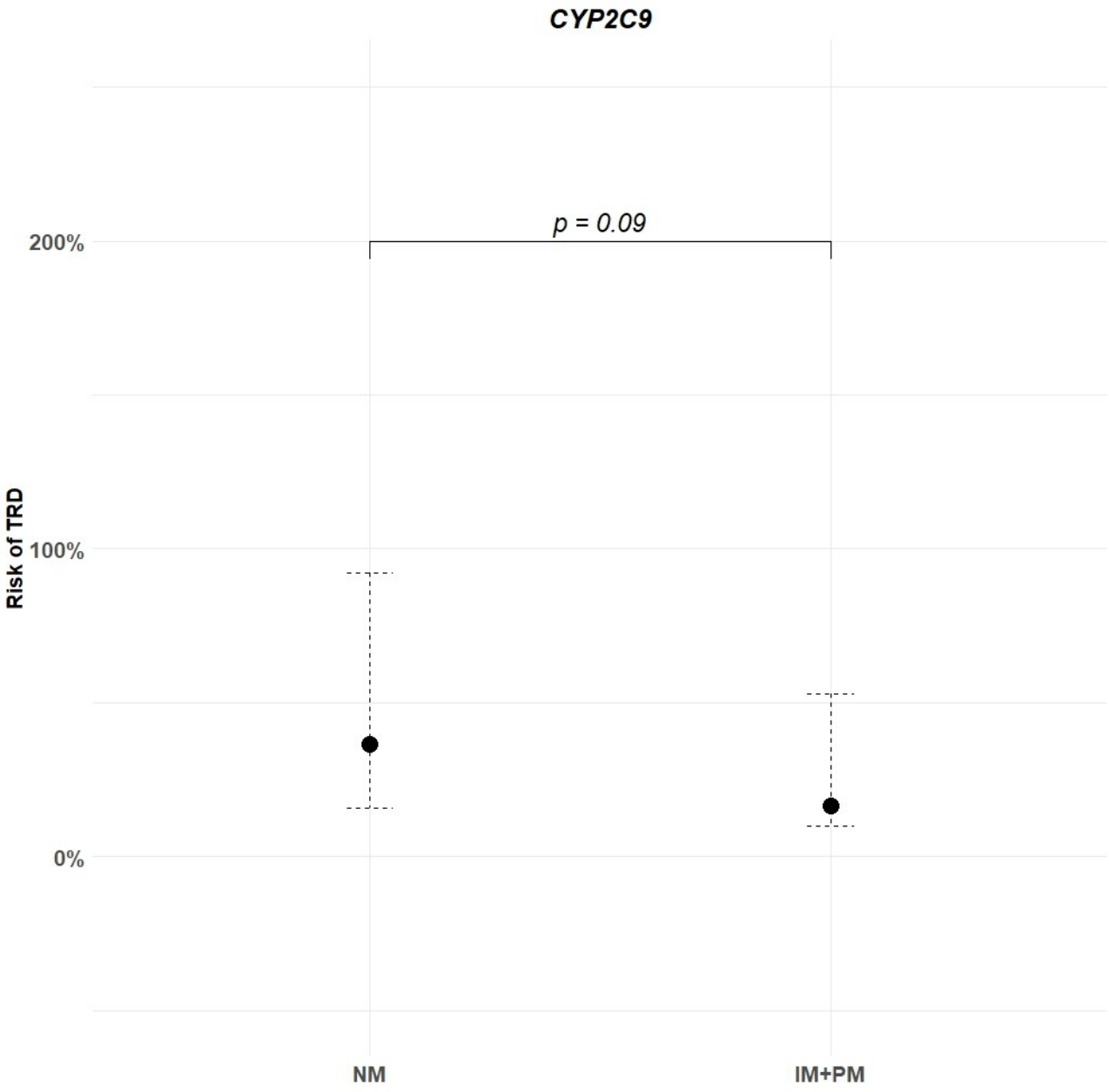

Figure 8: Main effect plot showing the predicted probability of TRD according to the *CYP2C9* metabolizer status. Dashed lines represent 95% Cis.

Additional comparisons did not highlight any other significant difference.

Association between genetic variants in SLC6A4 and response phenotype

Allele frequencies of 5-HTTLPR and rs25531 are reported in Table 14. No difference was found in allelic distribution between the two groups of response for each *SLC6A4* variant.

	TRD (N=84)	Responder (N=100)	Overall (N=184)	p-value
5-HTTLPR				
LL	24 (28.6%)	37 (37.0%)	61 (33.2%)	0.34
LS	45 (53.6%)	43 (43.0%)	88 (47.8%)	
SS	15 (17.9%)	20 (20.0%)	35 (19.0%)	
rs25531				
A/A	77 (91.7%)	86 (86.0%)	163 (88.6%)	0.23
A/G	7 (8.3%)	14 (14.0%)	21 (11.4%)	

Table 14: Allele frequencies of 5-HTTLPR and rs25531 in TRD and responder groups.

The impact of 5-HTTLPR and rs25531 on response phenotype was assessed using logistic regression as follows:

1. By comparing the risk of TRD in SS and LS patients with those of patients carrying LL genotype.
2. By comparing the risk of TRD in L carriers with those of non-carriers.
3. By comparing the risk of TRD in S carriers with those of non-carriers.

Similarly, the impact of A/G rs25531 SNP on response to AD drugs was assessed by evaluating the risk of TRD in A/A homozygotes compared to that of A/G heterozygotes. The G/G genotype was not observed in our MDD cohort. The responder group was considered as the reference in all comparisons.

No significant results emerged from the aforementioned analyses. However, carriers of at least one S allele (LS + SS) in 5-HTTLRP showed a trend for higher probability of TRD than non-carriers (LL) ($p = 0.09$, OR: 3.23, 95% CI [0.82, 12.78]). Interestingly, the risk increased in homozygotes for the S allele when compared with LL patients ($p = 0.07$, OR: 5.022, 95% CI [0.88, 28.74]) (Figure 9).

Because of the cumulative effect of rs25531 and 5-HTTLPR on *SLC6A4* transcriptional levels, also the potential impact of triallelic locus 5-HTTLPR/rs25531 on treatment outcome was evaluated. On the basis of previously published studies (Minelli et al. 2011; Bonvicini et al. 2010), 5-HTTLPR/rs25531 haplotypes were classified according to their effects on *SLC6A4* transcriptional levels as follows:

patients having two La alleles were placed in the “high SLC6A4 activity” group (L’L’); carriers of an active allele (La) and one allele with a reduced activity (Lg, Sa, or Sg) were placed in the “intermediate SLC6A4 activity group” (L’S’), whereas carriers of two alleles associated with reduced activity (Lg/Lg, Lg/S, or S/S) were assigned to the “low SLC6A4 activity” group (S’S’) (Table 15).

	TRD (N=84)	Responder (N=100)	Overall (N=184)	p-value
SLC6A4 activity groups				
L'L'	21 (25.0%)	32 (32.0%)	53 (28.8%)	0.18
L'S'	45 (53.6%)	40 (40.0%)	85 (46.2%)	
S'S'	18 (21.4%)	28 (28.0%)	46 (25.0%)	

Table 15: Distribution of 5-HTTLPR/rs25531 haplotypes according to the SLC6A4 activity groups in TRD and responder groups. L’L’ = high activity group (La/La); L’S’ = intermediate activity group (La/Lg, La/Sa, La/Sg); S’S’ = low activity group (Lg/Lg, Lg/S, S/S).

No significant differences emerged between TRD and responders based on SLC6A4 activity groups and also logistic regression analysis did not evidence any effect of genotype on AD response outcomes. However, a trend for association was observed between patients carrying a S’ allele or S’S’ genotype and TRD. Indeed, these patients showed an increased probability of TRD than L’L’ carriers ($p = 0.07$, OR: 5.34, 95% CI [0.88, 32.31]). A similar trend was also observed in carriers of at least one S’ allele compared to L’ homozygotes ($p = 0.09$, OR: 3.58, 95% CI [0.83, 15.50]) (Figure 9).

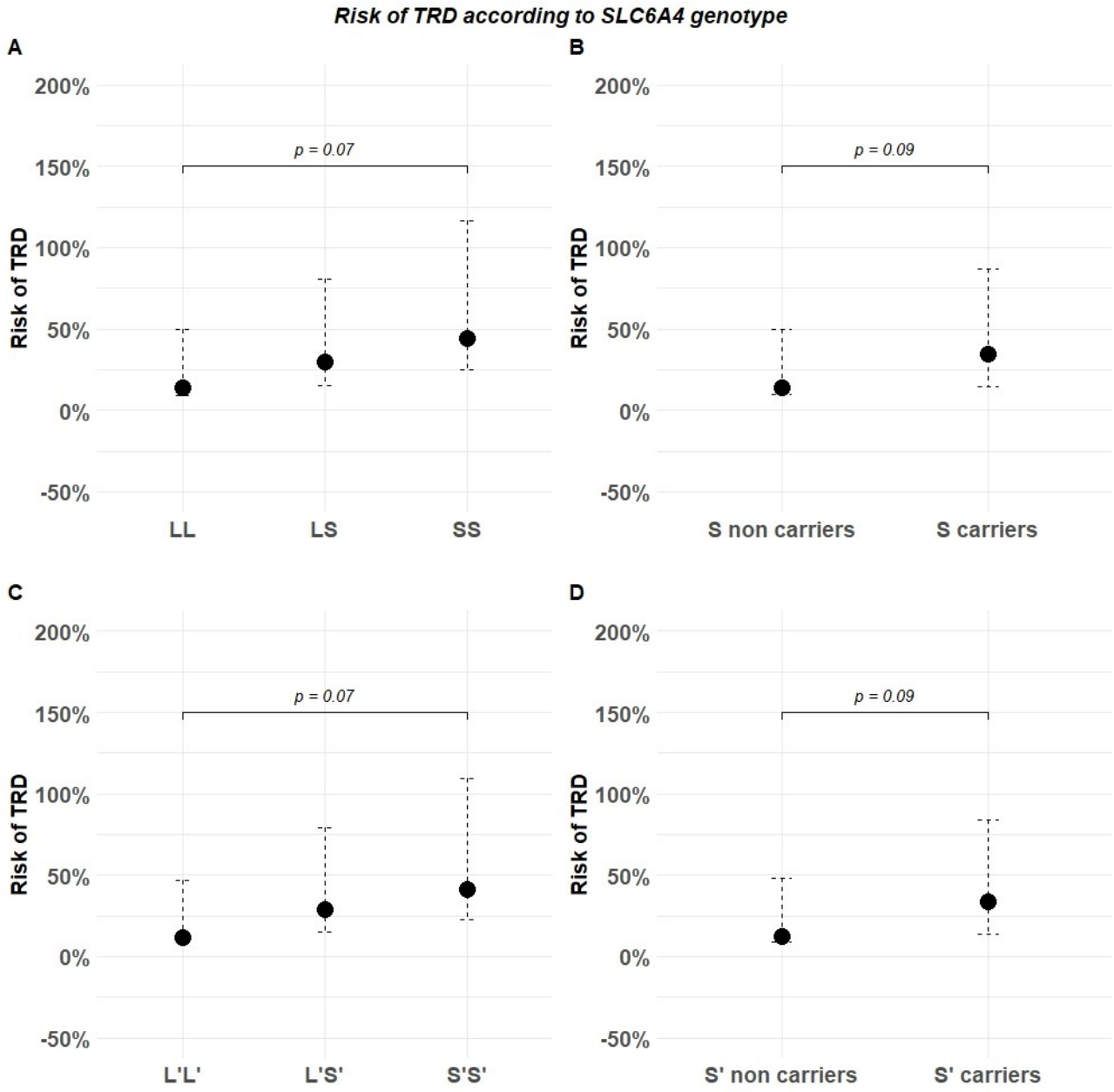

Figure 9: Main effect plot showing the predicted probability of TRD according to the genotype for 5-HTTLPR (A and B) and 5-HTTLPR/rs25531 haplotypes (C and D). Dashed bars represent 95% Cis.

7. Preliminary candidate gene association study to identify new genetic biomarkers of Treatment Resistant Depression

7.1. Aim of the work

The work described in Chapter 6 aimed at investigating the role of known AD pharmacokinetics- and pharmacodynamics-related genes in predicting TRD development. There are several genetic variants that, in spite of having been suggested as implicated in PGx of ADs in the literature are not usually considered when PGx test are developed, due to a lack of supporting evidence. However, these polymorphisms could potentially be implicated in TRD development. For this reason, we chose to analyse variants characterized by somewhat less robust evidence of association with AD response for their association with AD treatment outcome in a cohort of MDD patients classified as responders or TRD.

7.2. Material and methods

Study participants

Patients of Italian ancestry with a diagnosis of moderate to severe MDD according to the DSM-IV criteria were recruited at the Psychiatric Hospital "Villa Santa Chiara", Verona, Italy and at the IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli, Brescia, Italy.

Patients were included in TRD group when they failed to respond to two or more adequate AD trials with two or more different pharmacological classes and a TCAs, as reported in Stage III of Thase and Rush Staging Method (Thase and Rush 1997). On the contrary, the responder group included only patients who achieved response, in term of symptomatology reduction, after the first adequate AD trial.

All patients were screened for the following clinical and demographic features: gender, age of MDD onset, age at first AD trial, smoking, family history of psychiatric disease (depression, bipolar disease, anxiety disorder, schizophrenia, or other psychiatric disorder), alcohol abuse, and suicide.

Sample processing and DNA extraction

For each patient, gDNA was extracted from whole blood using QIAmp DNA blood Maxi kit (Qiagen) and evaluated with NanoDrop Spectrophotometer 2000 (Thermo Fisher Scientific) for quality and concentration. Samples were stored at -20° until use.

SNP selection and genotyping

SNPs evaluated in the study were selected from the PharmGKB database. Only those SNPs having a low level of evidence (level 3) of association with AD response in European individuals were included in the genotyping panel. In presence of SNPs in linkage disequilibrium, only one SNP was considered as representative for the locus.

Genotyping was conducted using a customized TaqMan™ OpenArray™ Genotyping plate and the QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems™), as described in the "Materials and methods" section of Chapter 5.

Statistical analyses

Statistical analyses were performed in R Studio (v. 4.1.3). Any significant difference in demographic and clinical features between TRD and responder groups was assessed by Kruskal-Wallis' test and Fisher's Exact test, depending on the type of variable (continuous or categorical, respectively). Normality of continuous variables was assessed through Shapiro-Wilk's test. Association analyses for genetic models were performed using *SNPassoc* package (González et al. 2007). Results of codominant models were further analysed through logistic regression (*glm* R function) in order to determine which genotype was associated with increased/reduced probability of TRD. Only associations having a p-value < 0.05 were considered as statistically significant.

7.3. Results

Clinical and demographic assessment

A total of 124 MDD patients - comprising 24 TRD and 100 responders – were included in the analysis. Females represented the 75.0% and 68.0% of patients in the TRD and responder groups, respectively, and the mean age of onset was 37.9 ± 15.4 in TRD and 40.3 ±15.9 in responders (Table 16).

	TRD (N=24)	Responders (N=100)	Overall (N=124)	p-value
Gender				
Male	6 (25.0%)	32 (32.0%)	38 (30.6%)	0.63
Female	18 (75.0%)	68 (68.0%)	86 (69.4%)	
Onset age (Years)				
Mean (SD)	37.9 (15.4)	40.3 (15.9)	39.8 (15.8)	0.46
Median [Min, Max]	37.5 [17.0, 69.0]	41.5 [11.0, 83.0]	40.0 [11.0, 83.0]	
Age at first treatment (Years)				
Mean (SD)	38.9 (15.7)	42.7 (15.0)	41.9 (15.2)	0.25
Median [Min, Max]	37.5 [18.0, 69.0]	44.0 [15.0, 83.0]	42.0 [15.0, 83.0]	
Familiarity for psychiatric diseases				
No familiarity	11 (45.8%)	28 (28.0%)	39 (31.5%)	0.26
Depression	10 (41.7%)	57 (57.0%)	67 (54.0%)	
Bipolar Disease	0 (0%)	6 (6.0%)	6 (4.8%)	
Anxiety Disorder	0 (0%)	2 (2.0%)	2 (1.6%)	
Schizofrenia	3 (12.5%)	7 (7.0%)	10 (8.1%)	
Other	0 (0%)	0 (0%)	0 (0%)	
Alcohol abuse				
No	23 (95.8%)	97 (97.0%)	120 (96.8%)	0.58
Yes	1 (4.2%)	3 (3.0%)	4 (3.2%)	
Smoking				
No	16 (66.7%)	63 (63.0%)	79 (63.7%)	0.82
Yes	8 (33.3%)	37 (37.0%)	45 (36.3%)	
Suicide				
No	23 (95.8%)	92 (92.0%)	115 (92.7%)	1
Yes	1 (4.2%)	8 (8.0%)	9 (7.3%)	

Table 16: Clinical and demographic features of TRD and responder groups.

Genetic association between PharmGKB level 3 SNPs and TRD development

A total of 29 SNPs have been evaluated in this analysis (details in Table 17). Each SNP was assessed for four genetic models: codominant (AA vs AB vs BB), dominant (AA vs AB + BB, where A is the most frequent allele in our patients' cohort), recessive (BB vs AA + AB, where B is the less frequent allele in our patients' cohort), and over-dominant (AA + BB vs AB). For the triallelic SNP rs2032582 the following models were considered: codominant (A/A vs C/C vs T/T), dominant (C/C + C/T + C/A vs T/T + T/A + A/A), recessive 1 (A carriers vs non-carriers), and recessive 2 (T carriers vs non-carriers). Responder group was considered the reference in all comparisons.

SNP	Gene
rs10248420, rs1045642, rs2235015, rs2032582	*ABCB1*
rs6265, rs962369	*BDNF*
rs41271330	*BMP5*
rs7569963, rs4675690	*CREB1 METTL21A*
rs2270007	*CRHR2*
rs1360780, rs3800373, rs4713916	*FKBP5*
rs948854	*GAL*
rs10975641	*GLDC*
rs5443	*GNB3*
rs1954787	*GRIK4*
rs915120	*GRK5*
rs6295	*HTR1A*
rs6311, rs7997012, rs9316233	*HTR2A*
rs11628713	*PAPLN*
rs1000940	*RABEP1*
rs11144870	*RFK*
rs3888190	*SH2B1*
rs2242446	*SLC6A2*
rs10879346, rs1487278	*TPH2*

Table 17: List of SNPs included in the genotyping panel.

Significant associations with TRD were detected for two SNPs in *HTR2A* (rs6311 and rs7997012), one in *GNB3* (rs5443), and one in *PAPLN* (rs11628713) (Figure 10). In particular, rs6311 was found to be significantly associated with TRD for the codominant (C/C vs C/T vs T/T, OR: 7.81, 95% CI [1.68, 36.34], OR: 2.93, 95% CI [0.53, 16.25], p = 0.005), dominant (C/C vs C/T + T/T, OR: 5.67, 95% CI [1.26, 25.54], p = 0.006) and over-dominant models (C/C + T/T vs C/T, OR: 4.14, 95% CI [1.57, 10.90], p = 0.003). The same three models were characterized by a significant association with TRD also for rs7997012 (codominant: G/G vs A/G vs A/A, OR: 0.23, 95% CI [0.07, 0.74], OR: 0.43, 95% CI [0.05, 2.81], p = 0.02; dominant: G/G vs A/G + A/A, OR: 0.26, 95% CI [0.09, 0.74], p = 0.007; over-dominant: G/G + A/A vs A/G, OR: 0.25, 95% CI [0.08, 0.79], p = 0.009) and rs5443 (codominant: C/C vs C/T vs T/T, OR: 0.22, 95% CI [0.07, 0.72], OR: 1.11, 95% CI [0.33, 3.69], p = 0.01; dominant: C/C vs C/T + T/T, OR: 0.40, 95% CI [0.16, 1.00], p = 0.05; over-dominant: C/C + T/T vs C/T, OR: 0.22, 95% CI [0.07, 0.68], p = 0.003). SNP rs11628713 was instead significantly associated with TRD under a recessive model (C/C + C/T vs T/T, OR: 5.00, 95% CI [1.32, 18.98], p = 0.02). A complete description of models for each SNP reaching the nominal significance threshold is reported in Table 18.

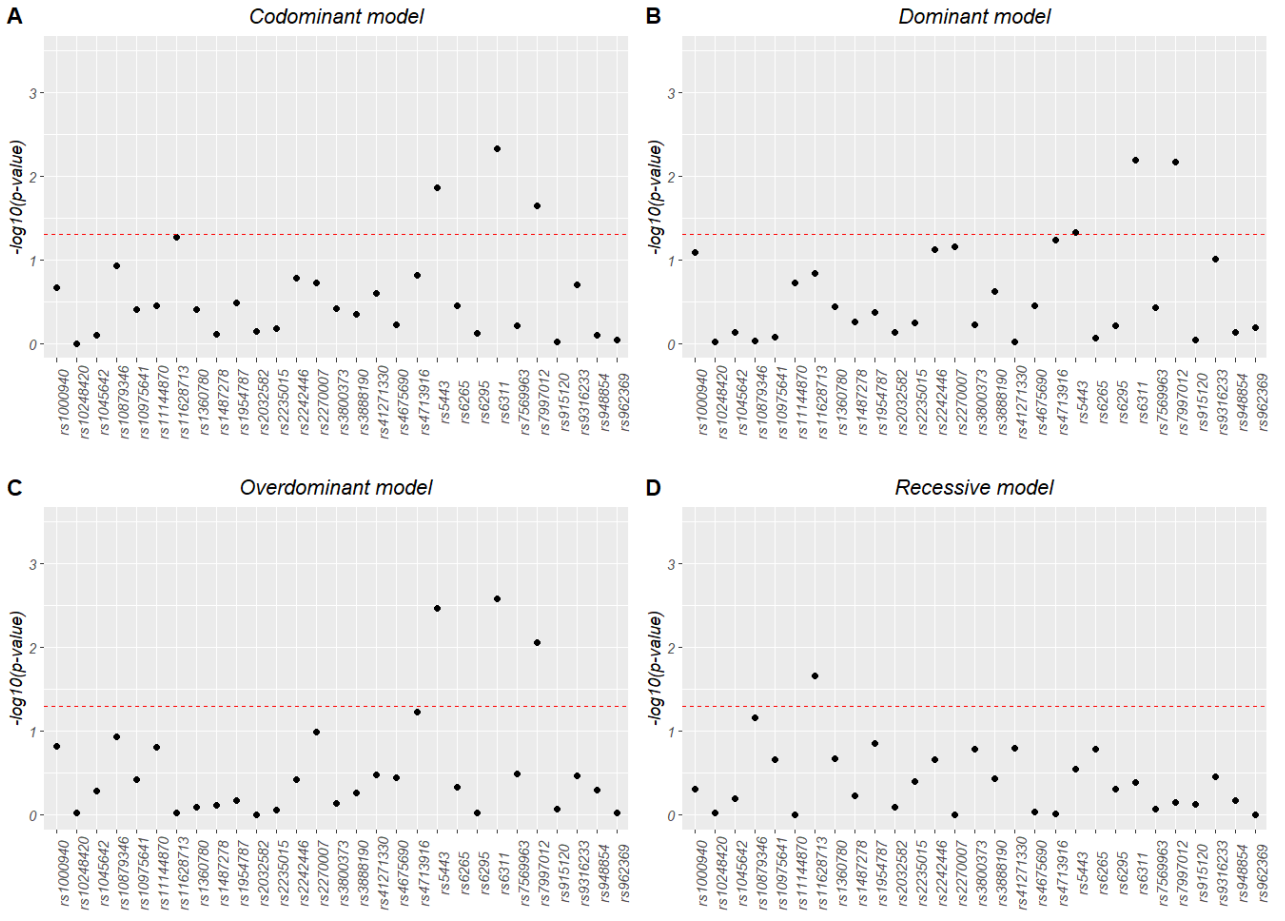

Figure 10: Manhattan plot of the -log10(p-values) for all the SNPs included in the analyses over all models. The dashed red line represents the nominal significance threshold ($p < 0.05$).

Gene	SNP	Genetic model		Responder	%	TRD	%	OR	95% CI	p-value	AIC
HTR2A	rs6311	Codominant	C/C	34	34.0	2	8.3	1.00		0.00	117.1
			C/T	37	37.0	17	70.8	7.81	1.68-36.34		
			T/T	29	29.0	5	20.8	2.93	0.53-16.25		
		Dominant	C/C	34	34.0	2	8.3	1.00		0.01	118.4
			C/T-T/T	66	66.0	22	91.7	5.67	1.26-25.54		
		Recessive	C/C-C/T	71	71.0	19	79.2	1.00		0.41	125.2
			T/T	29	29.0	5	20.8	0.64	0.22-1.89		
		Overdominant	C/C-T/T	63	63.0	7	29.2	1.00		0.00	116.8
			C/T	37	37.0	17	70.8	4.14	1.57-10.90		
HTR2A	rs7997012	Codominant	G/G	49	49.5	19	79.2	1.00		0.02	119.8
			A/G	44	44.4	4	16.7	0.23	0.07-0.74		
			A/A	6	6.1	1	4.2	0.43	0.05-3.81		
		Dominant	G/G	49	49.5	19	79.2	1.00		0.01	118.1
			A/G-A/A	50	50.5	5	20.8	0.26	0.09-0.74		
		Recessive	G/G-A/G	93	93.9	23	95.8	1.00		0.71	125.3
			A/A	6	6.1	1	4.2	0.67	0.08-5.88		
		Overdominant	G/G-A/A	55	55.6	20	83.3	1.00		0.01	118.5
			A/G	44	44.4	4	16.7	0.25	0.08-0.79		
GNB3	rs5443	Codominant	C/C	40	40.0	15	62.5	1.00		0.01	119.3
			C/T	48	48.0	4	16.7	0.22	0.07-0.72		
			T/T	12	12.0	5	20.8	1.11	0.33-3.69		
		Dominant	C/C	40	40.0	15	62.5	1.00		0.05	121.9
			C/T-T/T	60	60.0	9	37.5	0.40	0.16-1.00		
		Recessive	C/C-C/T	88	88.0	19	79.2	1.00		0.28	124.7
			T/T	12	12.0	5	20.8	1.93	0.61-6.13		
		Overdominant	C/C-T/T	52	52.0	20	83.3	1.00		0.00	117.3
			C/T	48	48.0	4	16.7	0.22	0.07-0.68		
PAPLN	rs11628713	Codominant	C/C	54	54.0	9	37.5	1.00		0.05	122.0
			C/T	41	41.0	10	41.7	1.46	0.54-3.93		
			T/T	5	5.0	5	20.8	6.00	1.44-24.98		
		Dominant	C/C	54	54.0	9	37.5	1.00		0.14	123.7
			C/T-T/T	46	46.0	15	62.5	1.96	0.78-4.89		
		Recessive	C/C-C/T	95	95.0	19	79.2	1.00		0.02	120.6
			T/T	5	5.0	5	20.8	5.00	1.32-18.98		
		Overdominant	C/C-T/T	59	59.0	14	58.3	1.00		0.95	125.8
			C/T	41	41.0	10	41.7	1.03	0.42-2.54		

Table 18: Results of association analyses for SNPs reaching the nominal significance threshold in at least one genetic model. AIC: Akaike Information Criterion.

Only the over-dominant model for rs6311 and rs5443 were significantly associated with response phenotype after false discovery rate correction (p_{adj} = 0.049).

In order to identify associations between each genotype and treatment outcome, a logistic regression analysis was performed for those SNPs reaching nominal significance in at least one genetic model. The responder group was considered the reference in all comparisons.

Compared to rs6311 C/T heterozygotes, C/C patients showed a small but significant reduction in probability of developing TRD (OR: 0.13, 95% CI [0.02, 0.49], p = 0.01). Patients carrying rs7997012 G/G had an increased probability of developing TRD than A/G heterozygotes (OR: 4.27, 95% CI: [1.47, 15.6], p = 0.01), as was the case for rs5443 C/C and T/T patients compared to C/T carriers (OR: 4.50, 95% CI [1.50, 16.8], p = 0.01; OR: 5.00, 95% CI [1.16, 23.1], p = 0.03). Finally, rs11628713 T/T patients showed an increased risk of TRD than C homozygotes (OR: 6.00, 95% CI [1.42, 26.1], p = 0.01) (Table 19).

	rs6311			rs7997012			rs5443			rs11628713		
SNP	**OR[1]**	**95% CI[1]**	**p-value**	**OR[1]**	**95% CI[1]**	**p-value**	**OR[1]**	**95% CI[1]**	**p-value**	**OR[1]**	**95% CI[1]**	**p-value**
rs6311												
C/T	—	—										
C/C	0.13	0.02, 0.49	0.009									
T/T	0.38	0.11, 1.08	0.083									
rs7997012												
A/G				—	—							
G/G				4.27	1.47, 15.6	0.014						
A/A				1.83	0.09, 15.3	0.61						
rs5443												
C/T							—	—				
C/C							4.50	1.50, 16.8	0.012			
T/T							5.00	1.16, 23.1	0.031			
rs11628713												
C/C										—	—	
C/T										1.46	0.54, 4.00	0.45
T/T										6.00	1.42, 26.1	0.014

[1] OR = Odds Ratio, CI = Confidence Interval

Table 19: Summary of logistic regression results. Nominally significant p-values are represented in bold.

8. Leukocyte telomere length in Treatment Resistant Depression

8.1. Aim of the study

The purpose of the analysis described in this chapter was to investigate leukocyte telomere length (LTL) in order to explore possible alterations in TRD. Telomeres are complexes of repetitive DNA sequences and proteins located at the end of each chromosome, with a protective function against DNA damage. During each cell cycle, about 50-200 bp are lost in telomeric regions, resulting in cell death when TL eventually becomes too short to enable further replications. Previous studies evidenced a shorter LTL in patients suffering from psychiatric disease including MDD, as a consequence of several pathological conditions such as inflammation and oxidative stress. However, only a limited number of studies investigated the role of LTL in AD response, with contrasting results. In this context, the aim of the present study was to evaluate the association between LTL and TRD in a cohort of patients suffering from MDD or bipolar disease (BD) in order to assess whether LTL could be involved in the genetic background that underlies resistance to treatment.

8.2. Material and methods

Study participants

A total number of 149 Italian TRD patients, of whom 126 MDD and 23 BD, were recruited at the Psychiatric Hospital “Villa Santa Chiara”, Verona, Italy, and included in the study. Differential diagnosis was confirmed using the SCID-I scale. Exclusion criteria were as follows: mental retardation and cognitive disorder; history of schizophrenic or schizoaffective disorder; primary diagnosis of personality, obsessive-compulsive, or post-traumatic stress disorders; comorbidity with eating disorder.

TRD was defined as the failure of at least two or more adequate trials with two or more different AD classes and to an adequate trial with a TCA, as defined by Thase and Rush Staging Method (Thase and Rush 1997).

Control individuals (n = 336) enrolled at the Lithium Clinic of the Clinical Psychopharmacology Centre of the University Hospital of Cagliari were ascertained as being without personal or family history of psychiatric conditions and included in the study.

Sample processing and DNA extraction

Genomic DNA was extracted from whole blood samples from the 149 TRD patients using the Gentra Puregene Blood kit (Qiagen), according to the manufacturer's instructions. DNA quantification and quality evaluation were performed by spectrophotometric analysis (NanoDrop 2000). For non-psychiatric controls, genomic DNA was extracted from peripheral blood leukocytes using the salting-out method (Lahiri and Nurnberger 1991).

Measurement of Leukocyte Telomere Length

Relative LTL was assessed according to the quantitative PCR method as previously described (Cawthon 2002). Samples were processed in triplicates both for the telomere (Tel) and for the single-copy gene (hemoglobin-b, Hgb) using Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX (Thermo Fisher) on a StepOnePlus ™ Real-Time PCR System (Thermo Fisher Scientific). Primer sequences were: Tel-1, 5'-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3', Tel-2, 5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3'; Hgb1, 5' -GCTTCTGACACAACTGTGTTCACTAGC-3', Hgb2, 5' -CACCAACTTCATCCACGTTCACC-3'. The PCR temperature conditions were 95 °C for 3 min followed by 28 cycles of 95 °C for 15 s and 60 °C for 1 min for Tel; 95 °C for 3 min followed by 32 cycles of 95 °C for 15 s and 60 °C for 1 min for Hgb. Specificity was assessed through the dissociation curve included in each plate. A control sample was included in each plate as a calibrator and LTL was calculated using the $2^{-\Delta\Delta CT}$ method where ΔΔCT = ΔCT sample − ΔCT calibrator and ΔCT sample = CT Tel − CT Hgb.

Statistical analysis

Normality of the data distribution was assessed using the Shapiro-Wilk's test. Association between LTL and quantitative or categorical variables was assessed using the Spearman's correlation test or the Mann-Whitney's U test, respectively. Analyses (adjusted for age) were conducted using partial correlation test or rank analyses of covariance (Quade's test), to evaluate potential association between quantitative or categorical variables, respectively. Since patients and controls were characterized by a significant age difference, a further analysis on a subsample matched for age was conducted using the Case Control Matching function in SPSS v.26, given that age is known to be correlated with LTL itself. A tolerance factor of three years was applied in order to minimize the loss of cases while still obtaining two groups of cases and controls that did not show a significant

difference in age. Stratified analyses were also conducted based on psychiatric diagnosis. Analyses were conducted with GraphPad Prism v. 9 and SPSS v.26.

8.3. Results

Comparison of LTL between TRD patients and controls

Following the exclusion of two outlier samples, 335 controls and 148 TRD patients (125 MDD and 23 BD) were included in the final analyses. Demographic characteristics of participants are reported in Table 20.

	Patients with TRD (*n* = 148)	**Controls (*n* = 335)**	**Statistics**	***p***
Age, median (IQR)	56 (20)	43 (22)	14,125 [a]	<0.0001
Gender (women, %)	67.6	53.1	8.76 [b]	0.004

Table 20: Demographic characteristics of TRD patients and controls. [a]Mann-Whitney U; [b]Pearson's Chi-Square. IQR = interquartile range.

LTL was negatively correlated with age (Spearman's coefficient = -0.25, p < 0.0001) and was not associated with gender (U = 25.835, p = 0.079) considering cases and controls combined (Figure 11).

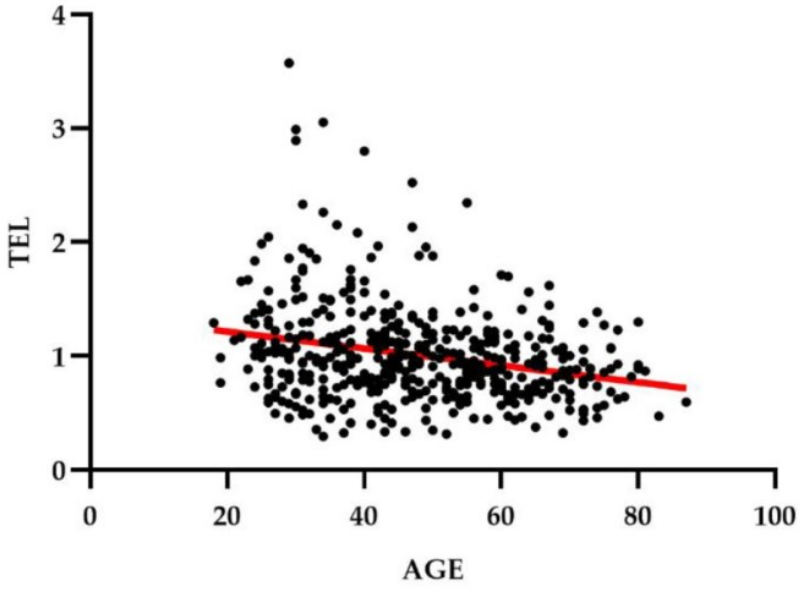

Figure 11: correlation between LTL and age in cases and controls combined.

Analyses revealed a shorter LTL in TRD patients, compared to controls (U = 13.015, p < 0.0001, Figute 12, Table 21). This LTL difference was significant also after adjusting for age using rank analysis of covariance (Quade's F = 49.17, p < 0.0001). When stratified for diagnosis, both MDD and BD patients showed a significantly shorter LTL compared to controls (MDD: U = 11.63, p < 0.0001, Quade's F = 35.18, p < 0.0001; BD: U = 1.39, p < 0.0001, Quade's F = 20.84, p < 0.0001).

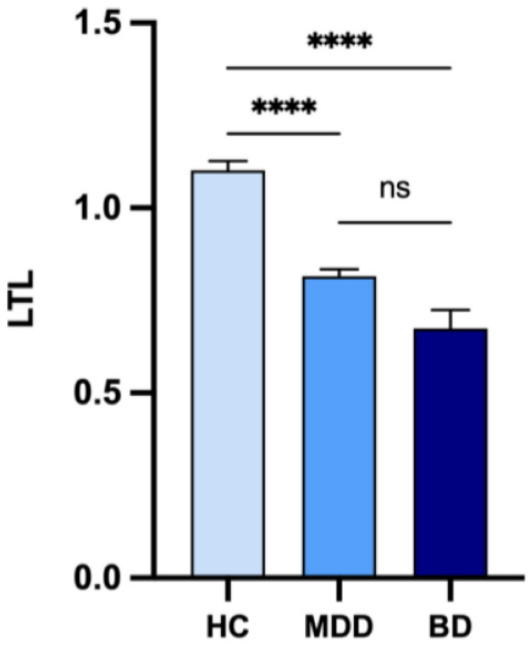

Figure 12: Bar-plot showing LTL in TRD patients stratified based on diagnosis and in controls.

		Unadjusted Analyses		Analyses Adjusted for Age	
	LTL, median (IQR)	U	*p*	Quade's F	*p*
Patients with TRD (*n* = 148)	0.77 (0.30)	13,015	<0.0001	49.17	<0.0001
Controls (*n* = 335)	1.03 (0.48)				

Table 21: Comparison of LTL between patients with TRD and controls. IQR = interquartile range.

LTL differences were further evaluated in age-matched subsamples comprising 147 TRD patients (median age: 56 years, IQR = 19 years) and 147 controls (median age: 54 years, IQR: 20 years). Both MDD and BD patients were characterized by a shorter LTL compared to controls (Table 22).

		Unadjusted analyses		Analyses adjusted for age	
	LTL, median (IQR)	U	p	Quade's F	p
Patients with TRD (n=147)	0.77 (0.30)	**5,567**	**< 0.0001**	**58.92**	**< 0.0001**
Controls (n=147)	1.03 (0.48)				
Patients with MDD (n=121)	0.79 (0.28)	**4,698**	**< 0.0001**	**47.91**	**< 0.0001**
Controls (n=147)	1.03 (0.48)				
Patients with BD (n=26)	0.70 (0.45)	**869**	**< 0.0001**	**22.81**	**< 0.0001**
Controls (n=147)	1.03 (0.48)				

Table 22: Comparison of LTL between TRD patients and controls in subsamples matched for age.

9. Discussion

Findings described in this work provide additional insights into the role of PGx in pharmacological treatment of MDD and they further support the implementation of PGx in clinical practice.

The first two studies herein reported (Chapter 4 and Chapter 5) focused on development and retrospective validation of combinatorial PGx test. Contrarily to single-gene PGx testing, which considers the impact of each gene in isolation on a given medication, combinatorial algorithms take into account the combined impact of variants in different genes on AD PGx, thus providing more accurate suggestions and a enabling to issue a report that is more rapidly applicable in the clinical practice (Winner and Dechairo 2015). Furthermore, the tool on which our PGx test is based is an open-source package that can be periodically updated in terms of both guidelines and drugs of interest, allowing to provide a report based on the most up-to-date literature evidence.

The PGx test described in Chapter 4 is currently under evaluation in an observational, prospective, double blind RCT (ClinicalTrials.gov Identifier: NCT04615234) aiming at the assessment of the role of our PGx test in improving response rate and reducing depressive symptoms in a cohort of Italian MDD patients who failed one AD treatment (Minelli et al. 2021). This trial officially started on February 1st, 2020 and should have originally been completed in approximately 30 months. However, because of the COVID-19 pandemic, patients' recruitment was delayed and data are not yet available for the analysis. For this reason, we decided to assess the effectiveness of our algorithm retrospectively, in a cohort of MDD patients on which information regarding response to pharmacological treatments were already available (Chapter 5). In accordance with literature evidence (showing a failure of the first AD in approximately 70% of MDD cases) about half of the patients included in our retrospective study needed a second treatment attempt due to a failure of the first one, either in terms of side effects or lack of therapeutic efficacy (Howland 2008). This trend has been observed throughout the entire study, with approximately 66% of patients receiving a further medication. Investigation of ADs' allocation in PGx reports revealed how the classical "trial and error" approach, based on clinical information and physician experience, may lead to the administration of an AD that is not suitable for the specific patient. Indeed, 15.88% of medication provided during the time of observation were placed in the "Red" recommendation category, indicating a gene-drug interaction most likely leading to treatment failure or presence of side effects. Our findings revealed that our PGx report could be potentially useful to avoid administration of unsuccessful treatments: indeed, retrospective analyses revealed the algorithm's ability to detect

gene-drug interactions potentially associated with treatment failure and revealed a 10.1-fold increased risk of failure in "Red" drugs. Basing on these results, PGx testing of patients before treatment start could help in avoiding the detrimental administration of drugs likely to fail, thus reducing the time to find an effective treatment. Furthermore, our algorithm seemed to be able to correctly predict unsuccessful treatments in particular during the second trial attempt, thus allowing us to expect positive results from its application in MDD patients who failed a previous AD treatment attempt. It is noteworthy that the aforementioned results refer to Italian patients with European ancestry and, due to the different allelic frequency of the variants of interest among the various biogeographic groups, the validity of our PGx test in other populations should be further assessed.

Studies described in Chapter 6 and Chapter 7 investigated the role of pre-selected AD PGx-related genes in TRD. In particular, the former focused on genes having a well-known role in PGx of ADs, providing interesting results. The evaluation of relationships between cytochromes metabolizer status and AD response phenotype revealed a higher probability of TRD in *CYP2C19* UM patients. To date, the association between lack of AD response and *CYP2C19* RM/UM has been detected only in a limited number of studies, also highlighting a reduced AD blood concentration in presence of the genotype associated with a high *CYP2C19* metabolism (Mrazek et al. 2011; Huezo-Diaz et al. 2012; De Vos, Van Der Weide, and Loovers 2010). Contrarily, the positive impact of a reduced *CYP2C19* metabolizer status on AD tolerance has been widely proven (Campos et al. 2022; Mrazek et al. 2011; Milosavljević et al. 2021; Chiara Fabbri, Tansey, et al. 2018). Although the role of *CYP2C19* on AD outcome is not fully clarified and contrasting findings stress the need for more investigations (Peters et al. 2008; Serretti et al. 2009), CPIC and DPWG consortia considered the available evidence as sufficiently robust to include some recommendations regarding AD dosage based on CYP2C19 metabolizer status in their guidelines (Hicks et al. 2015; Brouwer et al. 2021) and our data support these indications for the prevention of TRD.

The second remarkable result of this study concerns the role of *CYP2C9* on AD outcome. To date, some studies support an impact of *CYP2C9* gene variants in AD pharmacokinetics (Scordo et al. 2005; LLerena et al. 2004). Results emerged from our study are consistent with these findings, confirming an association between low CYP2C9 metabolic activity diplotypes and reduced risk of TRD.

Moreover, our data suggest a role for the two genetic variants in the promoter region of *SLC6A4* gene, 5-HTTLPR and rs25531, in TRD development. Although these results did not reach significance,

some suggestive associations emerged from our analyses are potentially relevant. In particular, interesting findings emerged for the 5-HTTLPR/rs25531 alleles correlated with a low *SLC6A4* transcriptional level, namely S allele, SS genotype and S'/S'S' haplotype, all characterized by a trend of association with an increased probability of TRD, in accordance with previous studies reporting a reduced AD response in patients with European ancestry carrying these variants (Fratelli et al. 2020).

Finally, our study did not reveal associations between the *CYP2D6* genotype and TRD. *CYP2D6* is one of the most investigated genes in pharmacokinetics of ADs and is included in all the commercial PGx test for MDD patients (Shalimova et al. 2021). Our results indicate no effect of *CYP2D6* on TRD development, but these findings might be explained by difficulties in determining the exact metabolizer status on the basis of genetic data. Indeed, in our genotyping panel we didn't evaluate some genetic factors that may influence *CYP2D6* haplotype definition, as the presence of rare or unknown genetic variants, events of hybridisation with the *CYP2D7* pseudogene, and structural alterations. Moreover, phenoconversion events could also occur for external factors, such as other drugs and diet (Taylor et al. 2020).

Regarding variants in the other candidate genes analysed, rs6311 and rs7997012, located in the gene encoding serotonin receptor 2A (*HTR2A*) have been associated with TRD in our MDD cohort. The *HTR2A* gene has been largely investigated as important target for many AD drugs (Ślifirski, Król, and Turło 2021) and several investigations revealed a role of variants in this gene in modulating AD response. In particular, carriers of at least one C allele in rs6311 showed a better treatment outcome after AD administration, whereas the T allele was more commonly observed in non-remitter patients compared with remitters (Choi et al. 2005; Kato et al. 2006). Similarly, the rs7997012 A allele has been found associated with a better response to citalopram (McMahon et al. 2006; Peters et al. 2009). Our results support these previously published findings and reveal a higher probability of TRD in rs6311 T carriers and a protective effect of the rs7997012 A allele against drug resistance.

Another SNP characterized by a significant association with TRD was rs5443, located in the *GNB3* gene. This gene encodes for the Gβ3 subunit of heterotrimeric G protein and is involved in the downstream signalling cascade following monoamine receptor activation. Our findings suggested a favourable role of rs5443 T allele in AD response, which is in accordance with previous studies showing higher SSRI response and remission rates in patients carrying the TT genotype (Q. Hu et al. 2015; Keers et al. 2011; Niitsu et al. 2013; Lin et al. 2009).

Finally, we reported an association between increased risk of TRD and T homozygosis in rs11628713, a SNP located in the *PAPLN* gene, encoding the proteoglycan like sulphated glycoprotein paplin. To date, the only study investigating a role of this SNP in MDD found an association with suicidal ideation following AD treatment (Laje et al. 2009).

The last study herein described (in Chapter 8) reveals an alteration of telomere length in TRD patients. Indeed, our findings highlight LTL to be shorter in TRD patients compared with non-psychiatric controls. Of note, this signal held also when analysing patients affected by MDD and BD separately. To date, different studies revealed a shorter telomere length in MDD, whereas only a few have been conducted to evaluate the association between LTL and AD response, with contradictory results (Ryan and McLoughlin 2020; Pisanu et al. 2020). Our study supports the observation of a reduced length of telomeres in patients resistant to AD treatments (Hough et al. 2016). The underlying mechanism that could link drug resistance to LTL reduction might be mediated by chronic inflammation that is often associated to TRD (Squassina, Pisanu, and Vanni 2019). However, further investigations with a control group of MDD responder patients are needed to better elucidate the association between telomere length and AD treatment outcomes.

Two limitations of the studies described above are worth mentioning. First, there is a relative degree of uncertainty in the attribution of the metabolizer status of cytochromes in Chapter 6. Indeed, as previously mentioned for *CYP2D6*, the presence of genetic variants not included in the genotyping panel or factors influencing the activity of CYP could hamper the correct attribution of the patients' metabolizer status. Second, the number of subjects included in the analyses is limited. In particular in Chapter 3 and 4, the smallest OR that may be detected with a power of 80% at the 5% level of significance is 2.59. This may have resulted in the inability to detect statistically significant associations between TRD and variants of interest, in particular after Bonferroni and false discovery rate corrections. For this reason, further analyses on larger cohorts are needed.

10. Conclusion

The work described in this thesis confirms the importance of PGx in MDD treatment and highlights the need for additional efforts in order to better understand the role of AD PGx in MDD.

These studies support the implementation of PGx tests in clinical practice to assist clinicians in prescribing an optimal AD treatment for each patient, with the long-term goal being the integration of precision medicine approaches in the psychiatry domain, leading to an optimization of MDD therapies. In this respect, results emerged from the retrospective analysis on the usefulness of our PGx test in MDD management are encouraging, and we hope they will be confirmed by the ongoing clinical trial.

Moreover, our data provided further evidence concerning the impact of genetic variants in TRD. Several PGx studies investigated the role of genetic variants in AD response, but only a minority focused exclusively on TRD. However, the comprehension of the TRD genetic background should be a priority, considering the strong impact of treatment resistance on patients' lives in terms of suffering, suicide risk, quality of life, and social costs. Findings from our study confirmed the role of well-known AD PGx genes, such as *CYP2C19*, on TRD and provided further insights into the involvement of additional PGx variants in treatment resistance. However, to date only a few literature evidence supports some association between genetic variants and the AD response described herein and, for this reason, replication on an enlarged cohort and through genome-wide data will be conducted in order to confirm or refuse such findings. Overall, this work highlights the need for further investigations including more genes in order to discover new biomarkers useful in preventing drug resistance and optimizing treatment.

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12. Appendix A

Publications in scientific journals

I. Magri C, **Vitali E**, Cocco S, Giacopuzzi E, Rinaudo M, Martini P, Barbon A, Grassi C, Gennarelli M. *Whole Blood Transcriptome Characterization of 3xTg-AD Mouse and Its Modulation by Transcranial Direct Current Stimulation (tDCS)*. Int J Mol Sci. 2021 Jul 16;22(14):7629. doi: 10.3390/ijms22147629. PMID: 34299250; PMCID: PMC8306644

II. Pisanu C, **Vitali E**, Meloni A, Congiu D, Severino G, Ardau R, Chillotti C, Trabucchi L, Bortolomasi M, Gennarelli M, Minelli A, Squassina A. *Investigating the Role of Leukocyte Telomere Length in Treatment-Resistant Depression and in Response to Electroconvulsive Therapy.* J Pers Med. 2021 Oct 27;11(11):1100. doi: 10.3390/jpm11111100. PMID: 34834452; PMCID: PMC8622097

III. Minelli A, Barlati S, **Vitali E**, Bignotti S, Dattilo V, Tura GB, Maffioletti E, Giacopuzzi E, Santoro V, Perusi G, Cobelli C, Magri C, Bonizzato S, Bocchio-Chiavetto L, Spina E, Vita A, Gennarelli M. *Clinical validation of a combinatorial PharmAcogeNomic approach in major Depressive disorder: an Observational prospective RAndomized, participant and rater-blinded, controlled trial (PANDORA trial).* Trials. 2021 Dec 11;22(1):896. doi: 10.1186/s13063-021-05775-8. PMID: 34895291; PMCID: PMC8665317

Conference abstracts

I. **E. Vitali**, V. Dattilo, S. Bignotti, G. Perusi, A. Squassina, L. Trabucchi, C. Cobelli, M. Manchia, G.B. Tura, M. Bortolomasi, M. Gennarelli, A. Minelli, P.0106 *Pharmacogenetic gene variants in treatment-resistant depression: preliminary results of a pilot study*. European Neuropsychopharmacology, Volume 53, Supplement 1, 2021, Pages S77-S78, ISSN 0924-977X, https://doi.org/10.1016/j.euroneuro.2021.10.107

II. **E. Vitali**, E. Maffioletti, A. Minelli, C. Bonvicini, M. Bortolomasi, G. Gainelli, L. Bocchio-Chiavetto, M. Gennarelli, P.264 *Association of single nucleotide polymorphisms in the 3' untranslated region of SLC1A2 with major depressive disorder and relative endophenotypes.* European Neuropsychopharmacology, Volume 40, Supplement 1, 2020, Pages S150-S151, ISSN 0924-977X, https://doi.org/10.1016/j.euroneuro.2020.09.198

Posters

I. **Vitali E.**, Giacopuzzi E., Cocco S., Maffioletti E., Grassi C, Gennarelli M. *Identification of peripheral gene expression biomarkers related to brain connectivity in a mouse model of*

Alzheimer's Disease stimulated with transcranial direct current stimulation. 2019 November 13-16. XXII SIGU National Congress, Rome

II. Minelli A., Magri C., Maffioletti E., Giacopuzzi E., Bortolomasi M., **Vitali E.**, Cattaneo A., Sacco C., Bonizzato S., Perusi G., Mazzelli M., Gennarelli M. *The effect of childhood trauma and trauma-focused psychotherapy on blood expression of the transcriptional factor MED22 in patients with major depressive disorder.* 2019 November 13-16. XXII SIGU National Congress, Rome

III. **Vitali E**., Maffioletti E., Minelli A., Bonvicini C., Bortolomasi M., Gainelli G., Bocchio Chiavetto L., Gennarelli M. *Association of single nucleotide polymorphisms in the 3' untranslated region of SLC1A2 with major depressive disorder and relative endophenotypes.* 2020 September 12-15. 33rd ECNP Congress, Virtual

IV. **Vitali E.,** Magri C., Giacopuzzi E., Cocco S., Grassi C., Gennarelli M. *Whole blood transcriptome characterization of the 3xTg-AD Alzheimer's Disease mouse model.* 2020 November 11-13. XXIII SIGU National Congress, Virtual Edition

V. **E. Vitali,** V. Dattilo, S. Bignotti, G. Perusi, A. Squassina, L. Trabucchi, C. Cobelli, M. Manchia, G.B. Tura, M. Bortolomasi, M. Gennarelli, A. Minelli. *Pharmacogenetic gene variants in treatment-resistant depression: preliminary results of a pilot study.* 2021 October 02-05, 34th ECNP Congress, Lisboa

VI. Magri C., **Vitali E.**, Cocco S., Giacopuzzi E., Rinaudo M., Martini P., Barbon A., Bagattini C., Fracassi C., Brignani D., Bortoletto M., Miniussi C., Binetti G., Grassi C., Gennarelli M. *Neuroplasticity transcriptional biomarkers in Alzheimer's Disease.* 2022 September 21. World Alzheimer Day, Brescia

VII. C. Galbiati, V. Dattilo, **E. Vitali**, M. Bortolomasi, M. Abate, L. Trabucchi, M. Gennarelli, L. Bocchio Chiavetto, A. Minelli. *MicroRNA changes after Electroconvulsive Therapy in Treatment Resistant Depressed patients*. 2022 October 07. World Mental Health Day, Brescia