UNIVERSIDADE D
COIMBRA

Paulo Ricardo Machado Magalhães

PHARMACOMETRIC EVALUATION OF THE CLINICAL IMPACT OF GENETIC POLYMORPHISMS OF THE CYTOCHROME P450 AND P-GLYCOPROTEIN IN THE PHARMACOKINETICS AND PHARMACODYNAMICS OF ANTIDEPRESSANT DRUGS: FLUOXETINE, PAROXETINE AND VENLAFAXINE

VOLUME 1

Tese de Doutoramento em Ciências Farmacêuticas, especialidade de Farmacologia e Farmacoterapia, orientada pelo Professor Doutor Amílcar Celta Falcão Ramos Ferreira, Professor Doutor Gilberto Lourenço Alves e Professor Doutor Adrián Llerena Ruiz e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Janeiro de 2020

Universidade de Coimbra

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The experimental work presented in this thesis was carried out at the Laboratory of Pharmacology of the Faculty of Pharmacy of the University of Coimbra (Coimbra, Portugal), at the Health Sciences Research Centre of the Faculty of Health Sciences of the University of Beira Interior (Covilhã, Portugal) and at Clinical Research Centre of the Extremadura University Hospital and Medical School (Badajoz, Spain), under the supervision of Professor Amílcar Celta Falcão Ramos Ferreira, Professor Gilberto Lourenço Alves and Professor Adrián Llerena Ruiz.

O trabalho experimental apresentado nesta tese foi realizado no Laboratório de Farmacologia da Faculdade de Farmácia da Universidade de Coimbra (Coimbra, Portugal), no Centro de Investigação em Ciências da Saúde da Faculdade de Ciências da Saúde da Universidade da Beira Interior (Covilhã, Portugal) e no Centro de Investigação Clínica da Área de Saúde de Badajoz (Badajoz, Espanha), sob a orientação do Professor Doutor Amílcar Celta Falcão Ramos Ferreira, do Professor Doutor Gilberto Lourenço Alves e do Professor Doutor Adrián Llerena Ruiz.

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À Sofia,

Aos meus Pais,

Ao meu Irmão,

Aos meus Avós,

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Um grande bem-haja e um muito obrigado!

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ABBREVIATIONS

A

ABCB1/MDR1	P-glycoprotein gene / Multidrug Resistance Protein 1 gene
AS	Activity Score
ASEC	Antidepressant Side-Effect Checklist
ASEC-GARSI	Global Adverse Reaction Severity Index
ASEC-PSEDI	Positive Side-Effect Distress Index
ASEC-PSER	Positive Side-Effect, Relevant Index
ASEC-PSET	Positive Side-Effect, Total Index
AUC	Area Under the Concentration-time Curve

B

BDNF	Brain-Derived Neurotrophic Factor

C

CAS	Combined Activity Score
CDPI	Combined CYP2D6-CYP2C9-CYP2C19 Drugs-Protein Interaction Score
CICAB	Clinical Research Centre of the Extremadura University Hospital and Medical School
CICS-UBI	Health Sciences Research Centre of the University of Beira Interior
C_{min}	Trough Concentrations
CNC	Centre for Neuroscience and Cell Biology of the University of Coimbra
COMT	Catechol-*O*-methyltransferase
CRP	C-Reactive Protein
CV	Coefficient of Variation
CYP	Cytochrome P450

D

DAD	Diode Array Detection
DAT	Dopamine Reuptake Transporter
DDV	*N,O*-Didesmethylvenlafaxine
DPI	Drugs-Protein Interaction Score

DSM	The Diagnostic and Statistical Manual of the American Psychiatric Association

E

EM	Extensive Metabolizer

F

FLD	Fluorescence Detection
FLU	Fluoxetine
[FLU]	Fluoxetine Concentrations
[FLU + NFLU]	Fluoxetine + Norfluoxetine Concentrations

G

GABA	Gamma-aminobutyric Acid
gEM	Genotype-predicted Extensive Metabolizer
gIM	Genotype-predicted Intermediate Metabolizer
gPH	Genotype-predicted Phenotype
gPM	Genotype-predicted Poor Metabolizer
gUM	Genotype-predicted Ultra-rapid Metabolizer

H

HAMD	17-item Hamilton Depression Rating Scale
HPA	Hypothalamus-Pituitary-Adrenal
HPLC	High-Performance Liquid Chromatography
HWE	Hardy-Weinberg Equilibrium

I

ICD	The International Classification of Diseases
IL	Interleukin
IR	Immediate Release
IS	Internal Standard

L

LC	Liquid Chromatography
LIC	Licarbazepine
LLE	Liquid-Liquid Extraction
LLOQ	Lower Limit of Quantification
LOD	Limit of Detection

M

MAF	Minor Allele Frequency
MAO	Monoamine Oxidase
MAOIs	Monoamine Oxidase Inhibitors
MEPS	Microextraction by Packed Sorbent
MS/MS	Tandem Mass Spectrometry

N

n (%)	Absolute Frequency (Relative Frequency)
NA	Not Available
NDV	*N-D*esmethylvenlafaxine
NET	Norepinephrine Reuptake Transporter
NFLU	Norfluoxetine
[NFLU]	Norfluoxetine Concentrations
NMDA	*N*-Methyl-*D*-Aspartate

O

ODV	*O*-Desmethylvenlafaxine
[ODV]	*O*-Desmethylvenlafaxine Concentrations
OR	Odds Ratio

P

PAR	Paroxetine
[PAR]	Paroxetine Concentrations
P-gp	P-glycoprotein
PM	Poor Metabolizer

Q

QC	Quality Control

R

R-FLU	*R*-Fluoxetine
R-NFLU	*R*-Norfluoxetine
rs	Spearman's Correlation
RT-PCR	Real-Time Polymerase Chain Reaction
R-VEN	*R*-Venlafaxine

S

SEM	Standard Error of the Mean
SERT	Serotonin Reuptake Transporter
S-FLU	*S*-Fluoxetine
S-NFLU	*S*-Norfluoxetine
SNPs	Single-Nucleotide Polymorphisms
SNRIs	Serotonin-Norepinephrine Reuptake Inhibitors
SPE	Solid-Phase Extraction
SSRIs	Selective Serotonin Reuptake Inhibitors
STAR*D	Sequenced Treatment Alternatives to Relieve Depression
S-VEN	*S*-Venlafaxine

T

T3	Triiodothyronine
T4	Tetraiodothyronine
TCAs	Tricyclic Antidepressants
TDV	*N,N,O-T*ridesmethylvenlafaxine
TeCA	Tetracyclic Antidepressant
TNFα	Tumour Necrosis Factor Alpha
TRH	Thyrotropin Releasing Hormone
TSH	Thyroid Stimulating Hormone

U

UBI	University of Beira Interior

UC	University of Coimbra
UHPLC	Ultra-High-Performance Liquid Chromatography
ULOQ	Upper Limit of Quantification
UV	Ultraviolet Detection

V

VEN	Venlafaxine
[VEN]	Venlafaxine Concentrations
[VEN + ODV]	Venlafaxine + *O-D*esmethylvenlafaxine Concentrations

W

wt	Wild-Type

X

XL-PCR	Extra-Long-Polymerase Chain Reaction
XR	Extended Release
χ2	Chi-Squared Test

5-HT	5-Hydroxytryptamine

FIGURES

TABLES

PUBLICATIONS

PUBLICATIONS

Magalhães P, Alves G, Llerena A, Falcão A. Venlafaxine pharmacokinetics focused on drug metabolism and potential biomarkers. *Drug Metabol Drug Interact.* 2014;29(3):129–41.

Magalhães P, Alves G, Rodrigues M, LLerena A, Falcão A. First MEPS/HPLC assay for the simultaneous determination of venlafaxine and *O*-desmethylvenlafaxine in human plasma. *Bioanalysis.* 2014;6(22):3025–38.

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ABSTRACT / RESUMO

ABSTRACT

More than half a century after the discovery of the first antidepressant drug, the drug treatment of depressive disorders is still a major problem, due to the high inter-individual variability and poor therapeutic outcomes.

Over the last years, the acknowledgement that a significant portion of the inter-individual variability in the drug outcomes is associated with genetic factors has provided the impetus for the pharmacogenetics research. However, pharmacogenetics alone has not been able to fully explain these clinical outcomes in a real-world setting, neither to provide clinically useful pharmacogenetic biomarkers for antidepressant drug therapy with the expected success.

Bearing these facts in mind, the present doctoral work aimed to conduct a comprehensive and integrated pharmacometric evaluation of the clinical impact of genetic polymorphisms of the cytochrome P450 (CYP) 2C9, CYP2C19, CYP2D6 and P-glycoprotein (P-gp), as well as of non-genetic factors, on the pharmacokinetics and pharmacodynamics of widely used antidepressant drugs [fluoxetine (FLU), paroxetine (PAR) and venlafaxine (VEN)], focusing on the identification of clinically relevant biomarkers. For that purpose, a multicentre clinical study was planned and developed in the real-world setting of treatment of depression, exploring an integrated pharmacogenetics and therapeutic drug monitoring approach, the so called *GnG-PK/PD-AD* study.

In a first phase, this doctoral work involved the development and validation of reliable bioanalytical tools to support the quantification of FLU + norfluoxetine (NFLU), PAR and VEN + *O*-desmethylvenlafaxine (ODV) planned in the *GnG-PK/PD-AD* study. Thus, two bioanalytical methods were properly validated: a high-performance liquid chromatography method coupled to fluorescence detection and using microextraction by packed sorbent (MEPS/HPLC-FLD) for the simultaneous quantification of VEN and ODV in human plasma and a MEPS/HPLC-FLD method for the quantification of FLU, NFLU and PAR in human plasma.

Afterwards, in the scope of the *GnG-PK/PD-AD* study, 182 patients with depression under treatment with FLU, PAR or VEN were recruited, clinically characterised and

submitted to the quantification of drug/metabolite plasma concentrations and genotyping of *ABCB1, CYP2C9, CYP2C19* and *CYP2D6* genes. Clinical outcomes of antidepressant drugs, including depression remission and adverse effects, were assessed by means of the *17-item Hamilton Depression Rating Scale* (HAMD) and the *Antidepressant Side-Effect Checklist* (ASEC), respectively.

Overall, the *GnG-PK/PD-AD* study provided a real-world clinical characterisation of Portuguese patients with depression and treated with FLU, PAR and VEN. Particularly, it demonstrated that the treatment of depression with these antidepressant drugs is frequently subject to a high inter-individual variability and poor clinical outcomes in the real-world setting.

Several genetic and non-genetic factors were identified as potential determining causes of these findings. Specifically, a high frequency of genetic polymorphisms and non-wild-type genotype-predicted phenotypes (gPHs) were found for the C*YP2C9, CYP2C19, CYP2D6* and *ABCB1* genes. Apart from these genetic factors, a co-morbid medical condition, polytherapy, a high risk of inhibition of P-gp, CYP2C9, CYP2C19 and CYP2D6 by drug-induced phenoconversion and, consequently, of drug-drug interactions were also found.

Moreover, potential genetic and non-genetic therapeutic biomarkers were identified for FLU. Specifically, genetically determined CYP2D6 activity was found as a predictor of FLU and NFLU concentrations, while the potential of the CYP2D6 to be inhibited by drug-induced phenoconversion was associated with a higher severity of depression. In turn, the *ABCB1 TTT*-haplotype was favourable to better clinical outcomes with FLU (lower severity of adverse effects and higher likelihood of remission) and the potential of the P-gp to be inhibited by drug-induced phenoconversion was associated with a worse tolerability profile (higher severity and number of adverse effects). Still, the presence of nervous system co-morbidities was associated with a higher severity of adverse effects, while aging and the female gender were associated with a higher severity of depression and a lower probability of remission.

Lastly, the present work offers real-world-based evidence which supports the necessity to change the mindset of pharmacogenetics and personalized medicine towards the integrated investigation of genetic and non-genetic factors and genotype-phenotype

associations in the drug treatment of depression, aiming the translation of the pharmacogenetics knowledge into clinical practice.

KEYWORDS: *Depression**,** Antidepressants, Real-world data, Cytochrome P450, P-glycoprotein, Pharmacogenetics, Therapeutic drug monitoring and Biomarkers.*

RESUMO

O tratamento farmacológico das perturbações depressivas é ainda um importante problema devido à elevada variabilidade inter-individual e aos maus resultados terapêuticos. Ao longo dos últimos anos, o reconhecimento de que uma grande parte da variabilidade inter-individual nos resultados terapêuticos está associada a fatores genéticos tem providenciado o ímpeto para a investigação farmacogenética. No entanto, a farmacogenética não tem sido capaz de explicar totalmente estes resultados clínicos em ambiente naturalístico, nem de providenciar biomarcadores farmacogenéticos clinicamente úteis para a terapia farmacológica antidepressiva, pelo menos com o sucesso que seria de esperar.

Considerando estes factos, o presente trabalho de doutoramento teve como objetivo levar a cabo uma avaliação farmacométrica integrada do impacto clínico de polimorfismos genéticos do citocromo P450 (CYP) 2C9, CYP2C19, CYP2D6 e da glicoproteína-P (P-gp), bem como de fatores não genéticos, na farmacocinética e farmacodinâmica de fármacos antidepressivos amplamente usados na prática clínica [fluoxetina (FLU), paroxetina (PAR) e venlafaxina (VEN)], visando a identificação de biomarcadores clinicamente relevantes. Com este objetivo, um estudo clínico multicêntrico foi planeado e desenvolvido em ambiente clínico naturalístico do tratamento da depressão, explorando uma abordagem integrada entre farmacogenética e monitorização farmacoterapêutica com base nas concentrações plasmática, designado por estudo *GnG-PK/PD-AD.*

Numa primeira fase foram desenvolvidas e validadas ferramentas bioanalíticas para suportar a quantificação da FLU + norfluoxetina (NFLU), PAR e VEN + *O*-desmetil-venlafaxina (ODV), planeada no estudo *GnG-PK/PD-AD.* A este nível, dois métodos bioanalíticos foram adequadamente validados: um método de cromatografia líquida com deteção de fluorescência e micro-extração em seringa empacotada (MEPS/HPLC-FLD) para a quantificação simultânea da VEN e da ODV em plasma humano e um outro método de MEPS/HPLC-FLD para a quantificação de FLU, NFLU e PAR em plasma humano.

Posteriormente, no âmbito do estudo *GnG-PK/PD-AD,* 182 doentes com depressão e tratados com FLU, PAR ou VEN foram recrutados, caraterizados clinicamente e

submetidos à quantificação das concentrações plasmáticas do fármaco/metabolito e à genotipagem dos genes *ABCB1, CYP2C9, CYP2C19* e *CYP2D6.* Os resultados clínicos dos fármacos antidepressivos, incluindo a remissão da depressão e os efeitos adversos, foram avaliados através da *Escala de Avaliação para Depressão de Hamilton de 17 itens* (HAMD) e da *Checklist de Efeitos Adversos dos Antidepressivos* (ASEC), respetivamente.

Globalmente, o estudo providenciou uma caraterização naturalística de doentes portugueses com depressão e tratados com FLU, PAR ou VEN. Em particular, este estudo demonstrou que o tratamento da depressão com estes fármacos antidepressivos é frequentemente sujeito a elevada variabilidade inter-individual e a resultados clínicos sub-ótimos em ambiente naturalístico. Vários fatores genéticos e não-genéticos foram identificados como causas potenciais para estes resultados. Especificamente, foi observada uma elevada frequência de polimorfismos genéticos e de fenótipos não *wild-type* nos genes C*YP2C9, CYP2C19, CYP2D6* e *ABCB1.* Para além destes fatores genéticos, os doentes apresentaram um estado clínico com múltiplas co-morbilidades, múltiplos tratamentos farmacológicos e um elevado risco de inibição da P-gp e do CYP2C9, CYP2C19 e CYP2D6 por fenoconversão induzida por fármacos, com o risco consequente de interações farmacológicas.

Adicionalmente, o estudo *GnG-PK/PD-AD* identificou potenciais biomarcadores genéticos e não genéticos para a FLU. Em detalhe, a atividade do CYP2D6 determinada geneticamente foi encontrada como um biomarcador das concentrações de FLU e NFLU, enquanto o potencial do CYP2D6 estar inibido por fenoconversão induzida por fármacos foi associado a uma maior severidade de depressão. Por sua vez, o haplótipo *ABCB1 TTT* foi favorável a melhores resultados clínicos com a FLU (menor severidade de efeitos adversos e maior probabilidade de remissão) e o potencial da P-gp estar inibida por fenoconversão induzida por fármacos foi associado a um perfil de tolerabilidade inferior (maior severidade e número de efeitos adversos). A presença de co-morbilidades do sistema nervoso central foi ainda associada a uma maior severidade de efeitos adversos, enquanto a idade e o género feminino foram associados a uma maior severidade de depressão e a uma menor probabilidade de remissão.

Por fim, o presente trabalho providencia evidência clínica naturalística que suporta a necessidade de modificar o estado da arte da farmacogenética e da medicina personalizada, em direção da investigação integrada de fatores genéticos e não genéticos

e associações genótipo-fenótipo no tratamento farmacológico da depressão, visando a translação do conhecimento farmacogenético para a prática clínica.

PALAVRAS-CHAVE: Depressão, Antidepressivos, Dados Naturalísticos, Citocromo P450, Glicoproteína-P, Farmacogenética, Monitorização Farmacocinética e Biomarcadores.

CHAPTER I

GENERAL INTRODUCTION

I.1 DEPRESSIVE DISORDERS

I.1 DEPRESSIVE DISORDERS

I.1.1 FROM MELANCHOLIA TO DEPRESSIVE DISORDERS – A HISTORICAL OVERVIEW

The experience of depressive disorders has plagued humans since the earliest documentation of human existence. Historical documents written by clinicians, philosophers, and writers during the story of humanity revel the long-standing existence of depressive disorders as a health problem and the consequent efforts to find effective ways to treat these disorders (1–3). The first references to depressive disorders appeared in the Mesopotamian texts in the second millennium B.C. with the description of melancholia, the first clinical description of what is currently called as major depression, clinical depression, unipolar depression or simply depression. At this time, melancholia was understood as a spiritual disorder, caused by spirits or demons, rather than a physical one and it was treated by priests (4,5). On the other hand, Roman and Greek clinicians early started addressing melancholia as a biological and a psychological disorder. Particularly, the understanding of the Greek physician *Hippocrates* about the mental health was the main milestone in that time. *Hippocrates* suggested that personality traits and mental disorders were related to balanced or imbalanced body fluids called humours. He described melancholia as a distinct disorder with specific mental and physical symptoms, namely "fear or sadness that last a long time, aversion to food, despondency, sleeplessness, irritability and restlessness". Curiously, this description is very similar to the currently accepted vision of depression (1–3). However, in the last years before Christ, the influence of *Hippocrates* declined, and the spiritual aetiology gained strength as the main explanation for mental illnesses, including melancholia. During the Middle Ages till Renaissance, religious beliefs, specifically from Christianity, dominated the vision of mental illness. With Renaissance, some physicians returned to the vision of *Hippocrates* (1,3).

During the 18th century, the progresses in the medical field stimulated the investigation of organic and physical causes of melancholia. The humoral theory of melancholia was increasingly challenged by the appearance of mechanical and electrical explanations (6). The term depression became to be used as a psychiatric symptom by the

French psychiatrist *Louis Delasiauve* in 1856, and by the 1860s it was appearing in the medical dictionaries to refer to a physiological and metaphorical lowering of emotional function (7). Despite melancholia has remained as the main term for clinical diagnostic in that period, the term depression was gaining popularity in the medical field, becoming a synonym by the end of the 19th century (1,2,6).

Later, particularly the works of *Sigmund Freud* (the father of psychoanalysis) and of the Swiss psychiatrist *Adolf Meyer* encouraged the introduction of a binary view of depression. *Sigmund Freud* argued that depression or melancholia could result from an objective (such as the death of a relative) and/or a subjective loss (such as the failure to achieve an important goal). *Adolf Meyer* put forward a mixed social and biological framework, emphasizing reactions in the context of an individual's life and defending that the term depression should be used instead of melancholia (1,3,4).

These important observations and discussions over the years culminated in the development of two major consensus-based classifications for mental disorders: *The Diagnostic and Statistical Manual of the American Psychiatric Association (DSM)* and *The International Classification of Diseases (ICD)* of the *World Health Organization*, which were independently developed, but converged in the late 1960s (1–3). The first version of the *DSM* (*DSM-I*) was introduced in 1952, where mental disorders, including the depressive ones, were classified into subtypes based on their supposed causes for the first time. Specifically, mental disorders were divided as organic, if the disturbance in mental functioning resulted from or was precipitated by a primary impairment of the brain function, or as reactive, if associated with a secondary impairment of the brain function in relation to a psychiatric disorder (1–3,8).

The 1950s were also golden years for the understanding of the pathophysiology of depression, as well as for its pharmacological treatment. In 1952, it was discovered that reserpine and the antituberculosis drug isoniazid modulate the endogenous levels of monoamine neurotransmitters in humans, relieving the symptoms of depression (1,9,10). These observations were the basis of the monoaminergic theory of depression, where depression is explained by an endogenous imbalance of key neurotransmitters in the central nervous system. Shortly after this significant finding, the practice of using medicines to treat mental disorders gained greater acceptance, opening the era of the antidepressant drugs (9,10).

The *ICD* was firstly issued in 1948, which passed to include a mental disorders section in its sixth revision (*ICD-6*) due to the development of the *DSM-I*, remaining identical in its seventh version (*ICD-7*). However, both systems (*DSM-I and ICD-7*) did not have a consensual international acceptance, once they were divergent in several aspects at the level of mental disorders. Thus, a close collaboration of the international community was put in place in the development of the *DSM-II* and *ICD-8*, in order to align both classifications (2).

Over the years the *DSM* and *ICD* systems have been revised and updated with new versions (1–3,8,11,12). Nowadays, *DSM-V* and *ICD-11* are the most updated international guidelines for the diagnostic of depressive disorders and present a high level of similarity in defining a major depressive episode (2,12). Both systems recommend the use of specific criteria (symptoms and features) for the diagnostic of a major depressive episode, which will be discussed below in section I. 1.3 (1–3,8,12,13).

I.1.2 EPIDEMIOLOGY

Depressive disorders are nowadays a serious public health concern given its increasing prevalence and negative burden on societies (14–17). According with the latest available *Global Health Estimates for depression and other common mental disorders* from the *World Health Organization*, depression affects over 300 million people worldwide, the equivalent to more than 4.4 % of the world´s population. Between 2005 and 2015, the global worldwide prevalence of depressive disorders increased around 18.4% (17). The prevalence of depressive disorders by world region, gender and age group is shown in Figures I.1.1 and I.1.2.

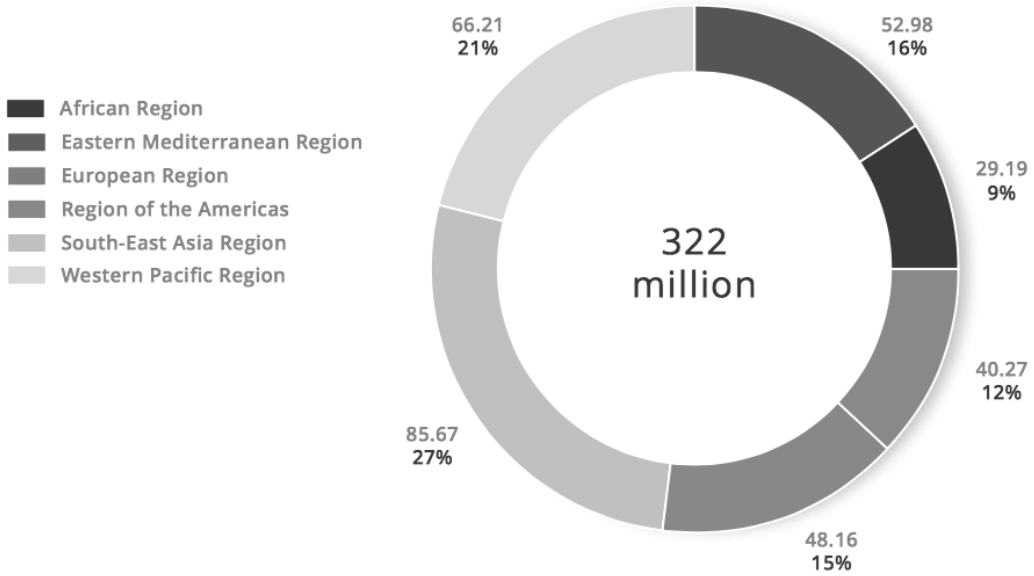


Figure I.1.1 Prevalence of depressive disorders by world region (millions and %) (17).

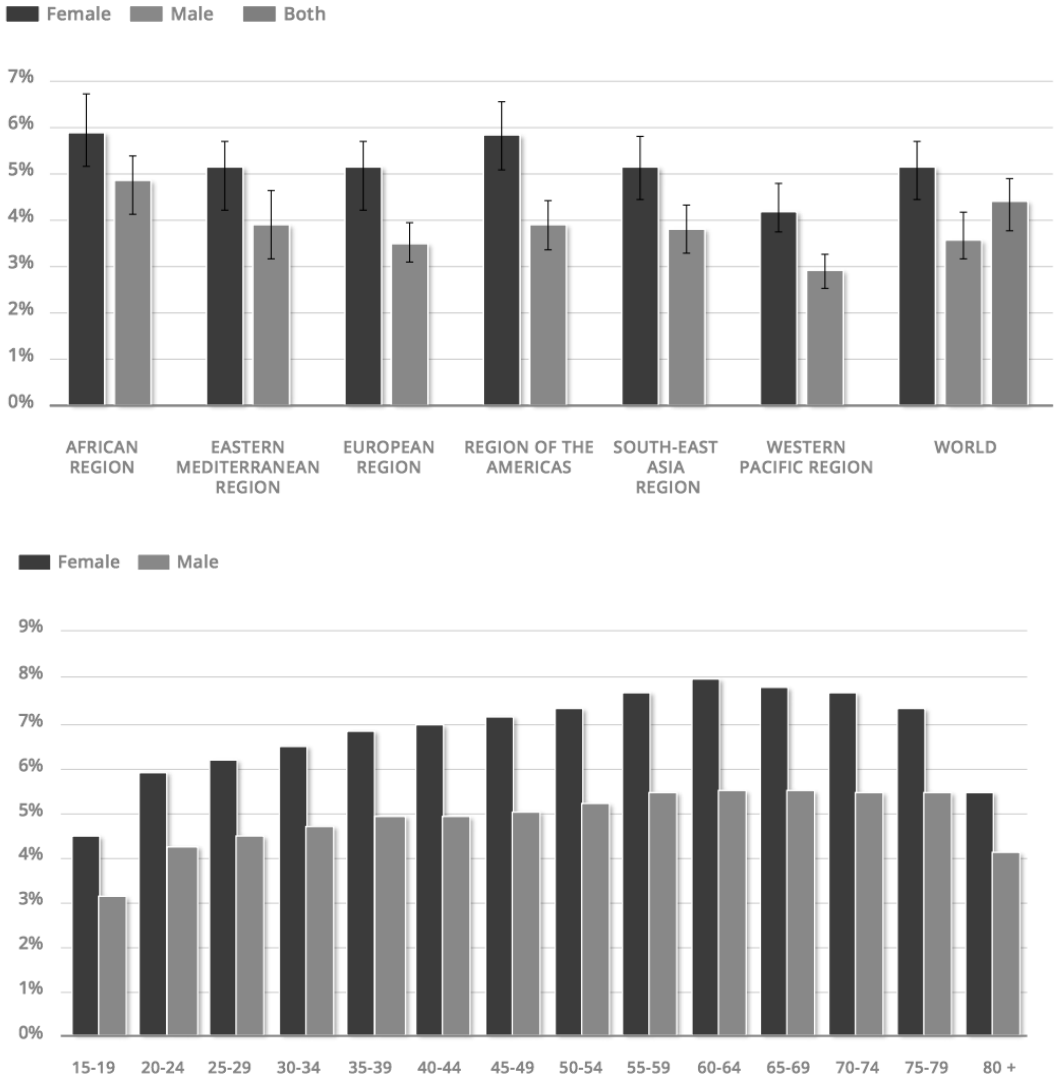


Figure I.1.2 Global prevalence of depressive disorders by world region, gender and age (% of population) (17).

Worldwide prevalence of depressive disorders varies from 2.6 % among males in the Western Pacific Region to 5.9 % among females in the African Region, with the European Region registering a prevalence higher than 5 % and 3 % of the total females and males, respectively (Figure I.1.2). In the European Region, Portugal was found to be the third country with the highest prevalence of depressive disorders, counting with a prevalence of approximately 5.7 % of its total population (17). Depression occurs about twice as often in women than in men with a worldwide and a lifetime prevalence around 5.1 % and 20-25 % in females and 3.6 % and 7-12 % in males, respectively (14,16). Prevalence rates vary by age, peaking in older adulthood between 55 and 74 years (Figure I.1.2).

Globally, the mean duration of a major depressive episode varies between 13 and 30 weeks. In outpatient care settings, only 25 % of patients remit within 6 months and more than half do not achieve the remission after 2 years. Even the patients in remission experience residual symptoms. Additionally, about 80 % of patients in remission experience at least one recurrence in their lifetime (16).

The burden of depressive disorders extends far beyond the disorder itself, which approximately accounts with 50 % of all psychiatric consultations and 12 % of all hospital admissions (16). Multiple studies have shown that depression increases the risk of diabetes *mellitus*, heart disease, stroke, hypertension, obesity, cancer, cognitive impairment and Alzheimer disease (16,18). In fact, depression is a common comorbidity of chronic medical disorders, namely asthma (27 %), atopic dermatitis (5 %), chronic obstructive pulmonary disease (24.6 %), gouty arthritis (20 %), rheumatoid arthritis (15 %), systemic lupus erythematosus (22 %), stroke (30 %), mild cognitive impairment (32 %) and oncologic and haematologic disorders (9.6-16.5 %) (14). On the other hand, depression increases the mortality risk. For instance, depression has been found as a risk factor of cardiovascular death (18). Major depressive disorder increases the mortality risk by 60–80 % with a contribution to all-cause mortality of about 10 % (16). At its worst, depression can lead to suicide. The mortality risk for suicide in depressed patients is more than 20-fold greater than in the general population. Approximately 800,000 people die worldwide due to suicide every year, being estimated that up to half of these suicides occur within a depressive episode. In 2015, suicide was into the top 20 leading causes of

death worldwide and the second leading cause of death between the 15 and 29 age group (15–18).

Apart from the morbidity-mortality risk, depression substantially impairs the individual's ability to function at work or school or cope with daily life (14–17). Depression is the leading cause of disability, including workplace disability, and the major contributor to the non-fatal health loss and disease burden worldwide (17,19,20). Indeed, depression frequently affects adults at working ages, increasing the risk of decreased workplace productivity, absenteeism, lowered income and, at worst, unemployment (17–20). Recent estimates have shown that 1 out of 10 workers have taken time off work due to depression in Europe, with a corresponding average time loss of 36 working days per depressive episode (20). In 2010, it was estimated that depression has a cost to the European economy around €92 billion, of which approximately €54 billion were related to indirect costs (such as absenteeism) (20,21).

I.1.3 SUBTYPES, CLASSIFICATION AND CLINICAL PRESENTATION

Depressive disorders are the group of mood disorders characterized by the persistent presence of sad, empty or irritable mood, accompanied by somatic and cognitive changes that significantly affect the individual's capacity to function. As previously mentioned, the clinical subtypes and classification of depressive disorders have been updated over the years by internationally consensually accepted guidelines for the diagnostic of mental disorders (1). Currently, the *DSM-V* (2013) is the international gold-standard and the most recent guideline for the classification and diagnostic of depressive disorders (12). Table I.1.1 below presents the *DSM-V* classification of depressive disorders. Globally, what differentiates the current subtypes of depressive disorders are issues related to duration, timing, or presumed aetiology, with major depressive disorder representing the classic condition of this group of disorders (12,22). The clinical presentation of these subtypes of depressive disorders is described below.

Table I.1.1 The Diagnostic and Statistical Manual of the American Psychiatric Association version V classification of depressive disorders (12).

1) Disruptive mood dysregulation disorder
2) Major depressive disorder
3) Persistent depressive disorder or dysthymia
4) Premenstrual dysphoric disorder
5) Substance/medication-induced depressive disorder
6) Depressive disorder due to another medical condition
7) Other specified depressive disorder
8) Unspecified depressive disorder

I.1.3.1 DISRUPTIVE MOOD DYSREGULATION DISORDER

Disruptive mood dysregulation disorder occurs among children between 6-18 years old, predominantly males, and it is characterized by a chronic, severe persistent irritability, which is clinically presented by two main types of manifestations. The first one, by frequent temper outbursts in response to common stressors over at least 1 year in at least two settings (such as at home and school). The second clinical manifestation is a persistent irritable or angry mood that is present between the severe temper outbursts, almost all day, nearly every day and noticeable by others. The onset of the disorder is frequently between 6-10 years old and patients typically present other mental co-morbidities with a wide range of disruptive behaviour, mood, anxiety, and even with autism (12,23).

I.1.3.2 MAJOR DEPRESSIVE DISORDER

Major depressive disorder is a complex and heterogeneous syndrome that covers a wide spectrum of symptoms. The course of the disease is pleomorphic, with considerable variation in remission and chronicity. The disorder may appear at any age, but the likelihood of onset markedly increases with puberty. According to *DSM-V*, major depressive disorder is clinically characterized and diagnosed by the presence for at least two weeks of five (or more) of the symptoms displayed in Table I.1.2 below (1,2,11,12).

Table I.1.2 The Diagnostic and Statistical Manual of the American Psychiatric Association version V criteria for the diagnostic of major depressive disorder (12).

1) **Depressed mood** most of the day, nearly every day
2) Markedly **loss of interest or pleasure** in all or almost all activities
3) Significant weight loss when not dieting or weight gain or decrease or increase in appetite
4) Insomnia or hypersomnia
5) Psychomotor agitation or retardation
6) Fatigue or loss of energy
7) Feelings of worthlessness or excessive or inappropriate guilt
8) Diminished ability to think or concentrate, or indecisiveness
9) Recurrent thoughts of death, recurrent suicidal ideation without a specific plan, or suicide attempt or a specific plan
Notes: at least one symptom is either: **1) Depressed mood** or 2**) Loss of interest or pleasure.**

Indeed, the essential feature of a major depressive episode is a period of at least two weeks during which there is either depressed mood or the loss of interest or pleasure in nearly all activities. The symptoms persist for most of the day, nearly every day, for at least two consecutive weeks. The episode is normally accompanied by clinically significant distress or impairment in social, occupational, or other important areas of functioning. Loss of interest or pleasure is nearly always present, at least to some degree. In some individuals, there is a significant reduction from previous levels of sexual interest or desire. Fatigue and sleep disturbance are present in a high proportion of cases. On the other hand, psychomotor disturbances are much less common, but are indicative of greater overall severity. Many individuals report impaired ability to think, concentrate, or make even minor decisions (12,16).

Several risk factors have been highlighted, including neuroticism (negative affectivity), adverse childhood experiences, chronic or disabling medical conditions, such as diabetes, morbid obesity and cardiovascular diseases. First-degree family members of individuals with major depressive disorder have a risk for major depressive disorder two- to four-fold higher than general population. The genetic contribution to major depressive disorder has been estimated to be approximately 35 % (12,16).

I.1.3.3 PERSISTENT DEPRESSIVE DISORDER (DYSTHYMIA)

Persistent depressive disorder, previously known as dysthymia, is a serious state of chronic depressive disorder, normally with less severity than major depressive disorder. The main associated feature is a depressed mood that occurs for most of the day for at least 2 years, or at least 1 year for children and adolescents. The diagnosis depends on the 2-year duration, which distinguishes it from episodes of depression that do not last 2 years. In addition, two (or more) of the following symptoms are present:

- poor appetite or overeating;
- insomnia or hypersomnia;
- low energy or fatigue;
- low self-esteem;
- poor concentration or difficulty making decisions;
- feelings of hopelessness.

Major depression may precede persistent depressive disorder and besides that, major depressive episodes may occur during persistent depressive disorder. Thus, an individual can be diagnosed with both depressive disorders. Persistent depressive disorder often has an early onset, that is in childhood, adolescence, or early adult life (12,24).

I.1.3.4 PREMENSTRUAL DYSPHORIC DISORDER

Premenstrual dysphoric disorder is a severe and disabling form of premenstrual syndrome that recur monthly during the luteal phase of the menstrual cycle. The essential clinical features of the premenstrual dysphoric disorder are the expression of mood lability, irritability, dysphoria, and anxiety symptoms. While the core symptoms include mood and anxiety symptoms, behavioural and somatic symptoms commonly also occur, namely loss of interest in usual activities, lack of energy, changes in appetite or food cravings, changes in sleep, and physical symptoms unique to the premenstruum. The woman may also suffer from difficulty in concentrating or a sense of feeling overwhelmed or out of control. These symptoms are present in most of the menstrual cycles during the past year and they have an adverse effect on work or social functioning. Symptoms

normally occur during the week before menses and remit after initiation of menses and display a comparable severity to those of major depression (12,25).

I.1.3.5 SUBSTANCE/MEDICATION-INDUCED DEPRESSIVE DISORDER

Substance/medication-induced depressive disorder is characterised by a prominent and persistent change in mood, with clear signs of depression or a marked decrease in interest or pleasure in daily activities and hobbies, during or soon after a certain substance/medication has been taken or withdrawal. The symptoms cause impairment in the day-to-day functionality of the individual. The diagnostic features of substance/medication-induced depressive disorder include the symptoms of a depressive disorder; however, the depressive symptoms are directly associated with the ingestion, injection, or inhalation of a substance and the depressive symptoms persist beyond the expected length of physiological effects, intoxication, or withdrawal period. Particularly, the symptoms develop during or within 1 month after use of the substance that is capable of producing the depressive disorder. Several substances/medications have been implicated in substance/medication-induced depressive disorder, including antiviral drugs (efavirenz), cardiovascular drugs (clonidine, guanethidine, methyldopa, reserpine), retinoic acid derivatives (isotretinoin), antidepressants, anticonvulsants, anti-migraine (triptans), antipsychotics, hormonal agents (corticosteroids, oral contraceptives, gonadotropin releasing hormone agonists, tamoxifen), smoking cessation drugs (varenicline), and immunological drugs (interferon) (12).

I.1.3.6 DEPRESSIVE DISORDER DUE TO ANOTHER MEDICAL CONDITION

Certain medical conditions can lead to a state of depression in an individual. Symptoms of depressive disorder due to another medical condition are similar to those found in the other depressive disorders. The major clinical feature of depressive disorder due to another medical condition is a prominent and persistent period of depressed mood or markedly diminished interest or pleasure in all, or almost all, activities, which is directly related to the physiological effects of another medical condition. Additionally, there is usually a temporal association between the onset, exacerbation, or remission of the

general medical condition and that of the mood disturbance, as well as the presence of features that are atypical for primary mood disorders. At this level, there are numerous medical conditions that have been linked to depression, such as stroke, Huntington's disease, Parkinson's disease, Cushing's disease, hypothyroidism, traumatic brain injury, chronic pain, cancer and multiple sclerosis (12,26).

I.1.3.7 OTHER SPECIFIED AND UNSPECIFIED DEPRESSIVE DISORDER

Other specified or unspecified depressive disorders are those cases where depressive symptoms yield clinically significant distress or impairment in social, occupational, or other important areas of functioning, however not meeting the full diagnostic criteria for any of the depressive disorders above described (12,16,22–26). Examples of other specified depressive disorders are:

- *Recurrent brief depression*: Concurrent presence of depressed mood and at least four other symptoms of depression for 2-13 days at least once per month (not associated with the menstrual cycle) for at least 12 consecutive months;
- *Short-duration depressive episode:* Depressed mood and at least four of the other eight symptoms of a major depressive episode associated with clinically significant distress or impairment that persists for more than 4 days, but less than 14 days;
- *Depressive episode with insufficient symptoms:* Depressed mood and at least one of the other eight symptoms of a major depressive episode associated with clinically significant distress or impairment that persist for at least 2 weeks, without meeting the criteria for any other psychiatry disorder (12).

Lastly, the unspecified depressive disorders are cases where the reason because the criteria are not met for a specific depressive disorder is not specified, including clinical presentations for which there is insufficient information to make a more specific diagnosis (12).

I.1.4 AETIOLOGY AND PATHOPHYSIOLOGY

I.1.4.1 OVERVIEW

To address and study the pathophysiology of depression is a unique challenge, not only by the clinical heterogeneity of depressive disorders, but also by the complex multifactorial aetiology, involving biological, psychological and social factors. Despite the tremendous progresses in neuroscience research over the last years, the pathophysiology of depression is not completely elucidated (16,27–33). However, several pathophysiological mechanisms have been implicated in the development of the disease, including altered serotonergic, noradrenergic, dopaminergic, and glutamatergic systems, increased inflammation, hypothalamic–pituitary–adrenal axis abnormalities, and decreased neurogenesis and neuroplasticity. Moreover, psychological and social factors have also demonstrated to affect the neurodevelopment, causing a biological predisposition to depression, while, in turn, biological factors can lead to psychological disorders as well (16,27–30,32,33).

Multiple hypotheses have been advanced to explain the aetiology and pathogenesis of depression; nonetheless, none of them alone has been able to fully explain the clinical symptomatology of the disease. Particularly, there is no consensus about the global model as depression should addressed at the pathophysiological level. Large discussion has occurred regarding if a unified model is the best approach to understand and explain depression (28,30–32). On the one hand, there are those who argued against a unified hypothesis of depression, because the pathophysiological mechanism and all theories of depression apply to only some types of depressed patients, but not others and, because depressive pathophysiology may vary considerably across the course of the disease (30,31). On the other hand, while the various biological mechanisms implicated in depression may appear unrelated, research has shown that all of these mechanisms are related and interconnected. For that reason, other authors have defended a unified model of depression, which incorporates all of these biological/pathophysiological mechanisms linked together, manifesting as a constellation of signs and symptoms which depict depression (28,32). This unified model considers the existence of several different endophenotypes of depression with distinct

pathophysiological mechanisms, but the core idea is that it is helpful to think of depression as one united syndrome (28). In fact, although depression presents several phenotypic expressions, they highly overlap between them in terms of clinical features and symptoms (section I.1.3) and, therefore, the investigation of the pathophysiology issues based on the *DSM* categorical diagnosis criteria has been difficult (28).

However, a consensual fact today is that depression is not only a disorder of the mind or the brain and there is no single hypothesis to explain it. The pathophysiology of the disease is currently assumed as a complex interaction of multiple aetiological factors, mechanisms, and hypotheses involving the whole body. These issues will be addressed in detail below.

I.1.4.2 MONOAMINES

Most fundamental brain functions depend on the presence and actions of various neurotransmitters at the pre- and post-synaptic membranes of the billions of neurons in the brain. Multiple evidence has supported the involvement of specific neurotransmitters in the pathogenesis of depression, namely the monoamines serotonin [also known as 5-hydroxytryptamine (5-HT)], norepinephrine and dopamine (16,28,32,34,35). These monoamine neurotransmitters were firstly implicated in the pathophysiology of depression after it has been observed that patients under treatment with the antihypertensive drug reserpine developed depression, since reserpine decreases the levels of these neurotransmitters. This monoaminergic involvement in depression was further reinforced with the discovery and understanding of the mechanisms of action of antidepressant drugs, which have shown to increase the monoamines levels in the brain (16,28,35).

Indeed, the brain contains vast numbers of noradrenergic, serotonergic and dopaminergic neurons. Noradrenergic neurons spread from the brain stem to almost all brain areas, where norepinephrine modulates the function of the prefrontal cortex, the processing of working memory and regulates behaviour and attention. Norepinephrine also mediates the acquisition of emotionally-arousing memories. In turn, serotonin innervates all brain areas and is the largest cohesive neurotransmitter system in the brain, while dopamine modulates reward and motivation functions, working memory and

attention (32). In other words, monoaminergic neurotransmission comprises the endobiological pathways to many clinical symptoms and signs of depression, such as low mood, vigilance, reduced motivation, fatigue, and psychomotor agitation or retardation. Differences in the central serotonin levels have been related to changes in behavioural and somatic functions observed in depressed patients, including appetite, sleep, sex, pain response, body temperature and circadian rhythm. Likewise, dopaminergic neurotransmission is responsible for cognitive outcomes, such as decision making and motivation, and abnormalities at this level have been associated with impaired motivation, concentration and aggression. Also, low norepinephrine levels mediate a broad spectrum of clinical depressive symptoms regarding sex, appetite, aggression, concentration, interest and motivation (28,32,34). Thus, all these observations led to the most relevant theory of depression, the monoaminergic hypothesis, which overall postulates that the underlying pathophysiological basis of depression is a direct and/or indirect impairment of the brain monoaminergic neurotransmission, with particular deficiency in the levels of serotonin and/or norepinephrine and/or dopamine (28,30,32,34).

In this context, several mechanisms for this monoaminergic impairment have been proposed, ranging from a decreased synthesis of the monoamines to an increased synaptic breakdown and even abnormalities in the receptor functions of the monoaminergic and/or related signal pathways. Figure I.1.3 schematically resumes the monoaminergic hypothesis and these putative mechanisms of monoaminergic impairment. For example, depressed patients with depletion of tryptophan have shown lower levels of serotonin and consequent reduced central serotoninergic function, because tryptophan is an essential amino acid for the synthesis of serotonin (28,34). In turn, the synaptic breakdown of monoamines occurs through metabolic degradation by monoamine oxidases and reuptake to the pre-synaptic compartment by protein transporters and, definitely, abnormalities in these processes may also modify the monoaminergic neurotransmission. For that reason, the inhibition of monoamine oxidase enzymes, as well as the inhibition of these transport proteins are mechanisms of action explored by antidepressant drugs. In fact, clinical studies have revealed an increased functional activity of monoamine oxidases in depressed patients (32,35).

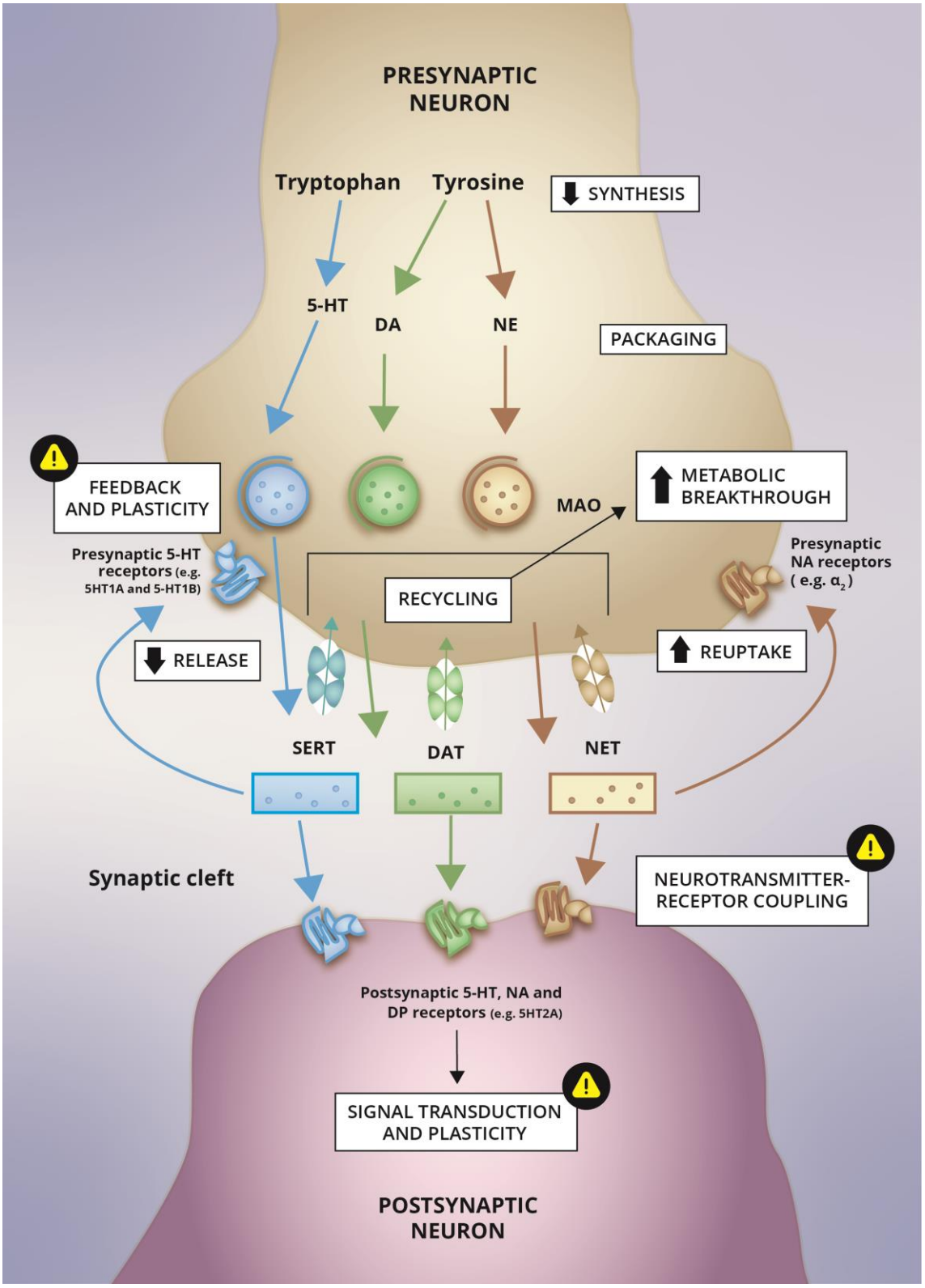


Figure I.1.3 Schematic representation of the monoaminergic hypothesis of depression and mechanisms involved. ↓, decreased; ↑, increased; 5-HT, serotonin; DA, dopamine; MAO, monoamine oxidase; NE, norepinephrine; DAT, dopamine reuptake transporter; NET; norepinephrine reuptake transporter; SERT; serotonin reuptake transporter. Abnormalities in the processes are identified with a yellow signal.

Also, transport proteins play a crucial role in the maintenance and regulation of nerve-nerve communications and monoaminergic transmissions, by enhancing or facilitating the pre-synaptic reuptake of the neurotransmitters. Through this process, transport proteins remove the neurotransmitters from the synaptic cleft, which, from one hand, terminates the action of the neurotransmitters and, on the other hand, decreases the metabolic breakthrough by monoamine oxidase enzymes. Hence, changes in the number and/or function of these transporters could also contribute to modify the central monoamine neurotransmission (32). These transporter proteins are specific to their respective neurotransmitter: serotonin reuptake transporter (SERT) for serotonin, norepinephrine reuptake transporter (NET) for norepinephrine, and dopamine reuptake transporter (DAT) for dopamine. The tricyclic antidepressants (TCAs) and nontricyclic serotonin-norepinephrine reuptake inhibitors (SNRIs) non-selectively block the SERT and NET, while the selective serotonin reuptake inhibitors (SSRIs) selectively block the SERT. Noteworthy, previous works have shown that genetic polymorphisms in the SERT and monoamine oxidase A genes (*SLC6A4* and *MAOA*, respectively) might increase the genetic susceptibility to develop depression, anxiety, stress or cognitive functions alterations (28,32,34,36).

Other potential mechanistic explanations assumed by the monoaminergic hypothesis for the impaired monoaminergic neurotransmission registered in depression are the abnormalities in the receptor functions. These abnormalities could result from impaired neurotransmitter-receptor coupling, normally due to decreased receptor affinity to neurotransmitters or decreased receptor numbers, or changes in the downstream signal transduction cascade. For example, alterations in the number and affinity of the type 1A (5-HT1A), type 1B (5-HT1B) and type 2A (5-HT2A) serotoninergic receptors and of the presynaptic α_2-noradrenergic receptors have been reported in the brain of depressed patients and associated with the pathophysiology of the disease (16,32,35). 5-HT1A receptors exert distinct functions depending on their brain location; they can work as auto-receptors regulating the release of serotonin from neurons or as heteroreceptors mediating the inhibition of non-serotonergic neurons. While 5-HT1A auto-receptors are known to undergo a rapid desensitization after activation, 5-HT1A heteroreceptors do not desensitize. In turn, 5-HT1B receptors contribute to the dynamic regulation of the serotonergic pathway. They are one of the main targets of

antidepressant drugs, which produce a notable reduction in the 5-HT1B receptors and a consequent increase in the release of serotonin. However, it is still not known whether 5-HT1B auto-receptors are subject to the same desensitization as 5-HT1A auto-receptors. Then, 5-HT2A receptor modulates the reuptake of serotonin and the release of other neurotransmitters. Studies have shown that depressed patients often have increased 5-HT2A receptor densities and that antagonism of the 5-HT2A receptor generates antidepressant-like effects by inhibiting the reuptake mechanism of serotonin and by modulating the release of other neurotransmitters (34). α_2-noradrenergic receptors inhibit the release of norepinephrine by negative feedback, reducing the noradrenergic neurotransmission. Concordantly, also, a super-sensitivity of the α_2-noradrenergic receptors has been linked to depression. Furthermore, alterations in main players of the signal transduction cascades, namely the G-protein and protein kinases, have been found at multiple sites of the cyclic adenosine monophosphate pathway in post-mortem brain tissue samples of depressed patients (16,32,35).

Overall, the serotoninergic and noradrenergic neurotransmissions have been the focus of research in the pathophysiology of depression. Nonetheless, serotonin, norepinephrine, and dopamine are all interrelated and their brain levels are co-influenced by each other (28,32,35). Dopamine has demonstrated an inhibitory effect on the release of norepinephrine from the locus coeruleus, while norepinephrine has an excitatory and inhibitory effect on dopamine release in the ventral tegmental area. Both norepinephrine and dopamine increase serotonin release from the dorsal raphe nucleus (28). Glutamate has also been implicated in mood regulation. The fact that ketamine, a *N*-methyl-D-aspartate (NMDA) receptor antagonist, acts as a potent and fast acting antidepressant has led to great interest in the glutamatergic system. Ketamine has been hypothesized to act through the antagonism of NMDA receptors in gamma-aminobutyric acid (GABA) interneurons, which reduces the inhibition of glutamate release in glutamatergic neurons and increases the neuroplasticity (28,32).

One of the problems of the original monoaminergic hypothesis is the fact that plasma concentrations of the antidepressant drugs and its pharmacological interaction with the therapeutic target occur almost immediately, whereas the antidepressant effect is normally observed only after a period of 3 to 6 weeks of treatment. This suggests that certain adaptive changes are occurring with the chronic administration of antidepressant

drugs. Thus, over the years, mechanisms such as downregulation of β-adrenergic receptors, desensitization of presynaptic α_2-adrenoceptors, increased postsynaptic serotonin receptors sensitivity, downregulation of 5-HT2 receptors, and desensitization of presynaptic 5-HT1A receptors have been proposed as consequent adaptive changes of the continuous antidepressant treatment. Globally, it has been argued that the downregulations/desensitisations of these receptors are the result of the sustained receptor activation secondary to continued elevations in monoamine levels after long-term antidepressant treatments (32,35). Importantly, it is also the fact that the SERT is highly regulated and undergoes adaptive changes upon SSRI treatment. *In vitro* studies indicate that following SSRI treatment, SERT undergoes internalization into the cytoplasm and that these internalized SERT molecules reappear on the cell surface of neurons after the cessation of treatment, suggesting that this process is transient and reversible (34).

Concluding, most evidence supporting the monoamine deficiency hypothesis is derived from the study of the molecular mechanisms of the antidepressant drugs currently available. However, their mechanisms of action are not fully understood and only approximately two thirds of patients achieve therapeutic response. This indicates that the pathophysiology of depression is not only a consequence of the monoaminergic imbalance, but also of other pathophysiological factors, as will be discussed below (16,35).

I.1.4.3 HYPOTHALAMUS-PITUITARY-ADRENAL AXIS AND STRESS RESPONSE

Stress and depression are commonly related. The influence of environmental stress and adverse life events on the development of depression is well documented, and most researchers report an excess of severely threatening life events before the onset of depression. Stressful life events can precipitate depressive episodes, limit the improvement and increase the probability of relapse (32). In this stress-depression relationship, the hypothalamus-pituitary-adrenal (HPA) axis is the key player, once it mediates the physiological response to stressful factors and has been implicated in the pathophysiology of depression (32,33,35,37).

A stressor evokes a biological response in the human body, which involves the release of hormones and other cellular mediators that may promote positive adaptation when the response is efficiently controlled, but which also may promote

pathophysiological processes when the response is dysregulated. Stress is integrated in the brain cortex and transmitted to the hypothalamus, leading to the HPA axis activation (32,35). HPA axis activity is regulated by adrenocorticotropic hormone-releasing factor and vasopressin, secreted from the hypothalamus, which in turn stimulates the pituitary to secret the adrenocorticotropic hormone, which finally activates the secretion of glucocorticoids (e.g. cortisol) from the adrenal cortex. Glucocorticoids control the activity of the HPA axis, by exerting a negative feedback on the release of corticotropin-releasing factor, vasopressin and adrenocorticotropic hormone. They are involved in the modulation of peripheral and central functions, such as metabolism, immunity, regulation of neuronal survival, neurogenesis, development of hippocampus, formation of new memories and the emotional assessment of events (32).

In fact, patients with depression have shown an intrinsic hyperactivity of the HPA axis and a consequent amplified response to stressors. Various types of abnormalities at the level of the HPA axis have been reported in depressed patients, namely, hypersecretion of adrenocorticotropic hormone-releasing factor and cortisol, dysfunctional glucocorticoid feedback mechanisms, inadequate HPA axis suppression in response to dexamethasone (dexamethasone suppression test) and impaired corticosteroid receptor signalling (28,35,37). The dexamethasone suppression test is the most specific measure of HPA axis overactivity. Dexamethasone administration suppresses adrenal corticosteroid production in normal subjects for 24 hours. Depressed patients have frequently demonstrated a negative dexamethasone suppression test, indicating overactivity or dysregulation of the HPA axis (28). Moreover, chronic high cortisol levels have been found in depressed patients and associated with the intensity, severity and risk of relapse of depressive symptoms, supporting the thesis of the HPA axis hyperactivity in depression (32,33). Several depressive signs and symptoms have been linked to the HPA axis dysfunction, such as excessive personal guilt and hopelessness, decreased appetite, weight loss, disrupted sleep, altered psychomotor activity and overactive response to psychological stressors. This is also aligned with the fact that patients with Cushing's disease, a clinical condition where there is a hyperactivation of the HPA axis, often suffer from depression. Furthermore, the trial use of anti-glucocorticoids to inhibit cortisol synthesis has experimentally produced antidepressant effects in both animal and human studies (32).

At this level, studies regarding the brain effects of glucocorticoids have elucidated why hypercortisolaemia may contribute to the development of depression. Glucocorticoids have demonstrated to change the activity of three main brain areas: medial prefrontal cortex, hippocampus, and amygdala. The medial prefrontal cortex is involved in executive functioning and the processing of emotion, the hippocampus is involved in memory and learning, and the amygdala is involved in the processing of emotion. Chronic stress has shown to decrease the activity of the medial prefrontal cortex, which leads to an inadequate processing of a negative effect. Furthermore, high levels of cortisol impair the ability of the hippocampus to adapt to a changing environment. In addition, chronic stress increases the response of the amygdala to stress and decreases the cognitive processing (38).

Resuming, the increased levels of glucocorticoids and the psychological stress may yield neurodevelopmental changes in the emotional-cognitive process, which may modify the way as the external environment is perceived, leading to a consequent disinterest in the external world, internal focus, and depressive symptoms. These facts provide a powerful framework for the co-integration of biological and psychological factors in the pathophysiology of depression (28,33).

I.1.4.4 THYROID HORMONES

The pathophysiology of neurodegenerative and psychiatric conditions, among them depression, has been associated with an imbalance in the thyroid hormones (32,33,38). Some symptoms of depression (e.g. weight loss, sleep disturbance and psychomotor agitation) have been associated with thyroid function abnormalities, and hypothyroidism has been linked to a depressive-like behaviour, which is successfully treated with adjuvant thyroid hormones (32,33).

The active forms of thyroid hormones, triiodothyronine (T3) and tetraiodothyronine (T4) are produced by the thyroid gland following stimulation by thyroid stimulating hormone (TSH) from the pituitary. THS secretion is modulated by the hypothalamic hormone thyrotropin releasing hormone (TRH) (32). T3 and T4 are of utmost importance for brain development, maturation and neurogenesis and regulate the overall metabolism in the human body (32,33,38).

Actually, multiple studies have related thyroid dysfunction with depressive symptoms and depressive disorders (32,33,38). However, the precise mechanism by which thyroid hormone abnormalities contribute to depression is not yet fully clarified and conflicting results exist (33,38). Despite this, animal studies have suggested that thyroid hormones increase the serotoninergic neurotransmission, which is aligned with the positive results of the hormonal supplementation with thyroid hormones in cases of refractory depression (33,38). On the other hand, other researchers have proposed that thyroid hormones may act as co-transmitters to norepinephrine in the adrenergic nervous system. Thus, it has been postulated that thyroid hormones dysfunction may indirectly produce symptoms of depression through the serotoninergic and/or noradrenergic systems (32).

I.1.4.5 IMMUNOLOGICAL FACTORS AND INFLAMMATION

Strong evidence supports that depression has an immunological/inflammatory trait, where cytokines assume the main role. Many immunological and inflammatory markers have been found to be elevated in depressed patients. These include interleukin (IL) IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, interferon gamma, C-reactive protein (CRP), tumour necrosis factor alpha (TNFα), and the chemokine monocyte chemoattractant protein-1 (28,29,32,33,39). In turn, the administration of pro-inflammatory cytokines and the induction of inflammation have demonstrated to induce symptoms similar to those observed in depressed patients (28,29,32,33,39). Consistent with the emerging recognition that inflammation may cause depression, an increased prevalence of autoimmune and inflammatory medical disorders has been registered in patients with depression (28,39). Large epidemiological studies have demonstrated that increased inflammation is a risk factor for the development of depression. On the other hand, other studies have found no correlation between inflammation and depression and the complete understanding of the involved mechanisms remains obscure (28,32,40). The explanation for this is that the relation between inflammation and depression is not straightforward. The revision of the literature suggests that inflammation and depression are complexly linked by diverse mechanisms and that an imbalance in the pro-

inflammatory and anti-inflammatory players may contribute to the pathogenesis of depression (28,32,33,38).

In this context, the observation that exposure to a psychosocial stressor can activate an inflammatory response in humans was the breakthrough to link inflammation to depression. As discussed above, stress and depression are intimately related, and apart from the overactivation of the HPA axis, stress has been associated with inflammation. A pro-inflammatory state and hyperactivity of the HPA axis have been of the most consistent biological findings in depression and are often associated (39,40). These findings are particularly enigmatic, especially considering the classic notion that high levels of cortisol have an anti-inflammatory action, and therefore the coexistence of inflammation and hypercortisolaemia in the same diagnostic group appears counter-intuitive (40). Nonetheless, glucocorticoids can be pro-inflammatory as well. Glucocorticoids increase inflammation by permitting catecholamine activation of immune cells and by causing white blood cells to leave circulation and travel to tissues. In addition, glucocorticoids have shown to stimulate the release of cytokines by macrophages (28).

In fact, pre-clinical and clinical findings have shown the existence of a central inflammatory response in human depression (neuroinflammation) that is primarily driven by peripheral inflammatory events (39). Overall, an increase in pro-inflammatory cytokines results in a lack of neuronal plasticity, eventual neurodegeneration and reduced neurogenesis, which may alter relevant neurocircuits for the control of the behaviour and mood, namely in terms of motor activity, motivation, anxiety and alarm reactions (32,33,40). At a molecular level, cytokines upregulate the HPA axis, which, as mentioned above, potentiates the inflammation as well. This explains the concomitant finding of inflammation and overactivation of the HPA axis in depressed patients (28,39,40). Finally, cytokines and glucocorticoids have shown to decrease monoamines by reducing their synaptic availability for neurotransmission through the increasing of their synaptic reuptake or the decreasing of their synthesis. Also, pro-inflammatory cytokines contribute to increase glutamate both within and outside the synapse cleft, which leads to increased excitotoxicity, decreased production of the brain-derived neurotrophic factor (BDNF) and consequent decreased neurogenesis, given that BDNF fosters neurogenesis (28,39).

Taken together, these facts support the notion that cytokines mediate neuroinflammatory effects on the brain, which lead to changes in the main neurotransmitters and brain neurocircuits associated with depression (39).

I.1.4.6 REDUCED NEUROPLASTICITY AND NEUROGENESIS

The brain possesses remarkable plasticity, able to rapidly create and eliminate synapses as well as to alter functional circuits in adaptation and learning. Neurogenesis in adult individuals involves the generation of entirely new neurons and neuronal connections in the dentate gyrus of the hippocampus and the sub-ventricular area of the lateral ventricles (28,32). In line with the depression-induced structural and functional changes, neurogenesis is essential for restoration of brain structure and function, namely at the level of hippocampal and, therefore, in learning and adaptation process which seems to be deficient in depression (28,32,33). Thus, it has been proposed that a lack or reduction in adult neurogenesis capacity may contribute to depression. One of the molecular factors needed for healthy neuroplasticity and neurogenesis is the BDNF. BDNF is a neurotrophin that promotes the survival of existing neurons and encourages the growth and differentiation of new neurons and synapses (28). The finding that serum levels of BDNF are reduced in patients diagnosed with depression implies a possible role of BDNF in the pathophysiology of depression. Accordingly, we previously discussed that inflammation reduces the neurogenesis and neuroplasticity by decreasing the level of BDNF (28,32). The expression of BDNF is believed to be halted by chronic stress and normal levels of this growth factor have been attained after a successful treatment with antidepressant drugs (33).

Indeed, many antidepressant drugs also seem to increase the brain neurogenesis and neuroplasticity (28,32). This finding has led to the suggestion of a network hypothesis of depression. This hypothesis advocates that antidepressant drugs restore a juvenile-like plastic state in which a depressed individual may alter networks in response to external signals. Despite the increase in the monoamine levels to be essential for the antidepressant effects, this may not be enough to directly improve mood; they probably increase the brain plasticity, which allows an individual to adapt and change. Thus, rather than a simple increase in the level of neurotransmitters, antidepressants possibly cause

longer lasting neuroadaptive changes in the brain. This is consistent with the fact that antidepressants take at least 2-3 weeks to exert their therapeutic actions (28,32,33).

I.1.4.7 GENETIC FACTORS

Despite the negative results reported by some studies (41–44), several genes have been associated with the development of depression (32,38). One example is the gene encoding the 5-HT1A, where genetic variants associated with a higher expression of the receptor within platelets were found in depressed patients compared to controls. At this level, decreased concentrations of serotonin were reported, suggesting that increased 5-HT1A expression inversely correlated with serotoninergic activity via a negative feedback mechanism (45). Another example is the HPA axis hyperactivity, which, as previously mentioned, seems to be involved in the pathogenesis of depression due to intrinsic abnormalities (46). On this matter, a longitudinal study found significant associations between several single-nucleotide polymorphisms (SNPs) of the angiotensin-converting enzyme and the risk of late-life depression (47). Genetic polymorphisms in the *FKBP5* gene, a relevant gene for immune regulation, have also been associated with the development of depression (48,49). In turn, a higher expression of the dopamine receptor gene *DRD4* has been reported in depressed individuals (50). Worthy of mention is an extensive genome-wide association meta-analysis that recently reported strong associations between depression and 42 sets of genes encoding for proteins which are known targets of antidepressants drugs and, therefore, which may contribute to explain the therapeutic efficacy of antidepressants (51). Nonetheless, no genetic factor is clinically approved as biomarker of depression up to now (32,38).

I.1.4.8 FINAL REMARKS

The current knowledge of the pathophysiology of depression shows that genetic, immunological, environmental and endocrine factors alone are not able to explain the pathogenesis of the disease. Contrarily, depression is a complex disorder in which these factors are linked and interact between them in the clinical development of the disease.

At this level, the HPA axis seems to be the major neurobiological link between such factors (28,32,35).

Briefly, environmental stressors and heritable genetic factors, acting through immunologic and endocrine responses, initiate structural and functional changes in many brain regions, which results in dysfunctional neurogenesis and neurotransmission and clinically manifests as the combination of symptoms observed in depression. Naturally, the response to environmental stressors is influenced by genetic components. Environmental stressors activate the HPA axis and it leads to hypercortisolaemia. This causes, by direct and indirect action, structural and functional changes in specific brain areas, which have been related to the development of psychological, cognitive, physical and emotional symptoms of depression (prefrontal cortex, hippocampus and amygdala). Particularly, stress-induced high glucocorticoid concentrations have been associated with the decrease of brain neurogenesis and with relevant changes in the brain neurotransmission. Moreover, cytokines also interact with the HPA axis. Increased pro-inflammatory cytokines reduce monoaminergic neurotransmission and brain neuroplasticity/neurogenesis, contributing to the development of depression. On the other hand, pro-inflammatory cytokines upregulate the HPA axis, which leads to a consequent hypersecretion of cortisol. However, it is also plausible that psychological or environmental stressors act through the HPA axis principally by increasing hypothalamic corticotropin-releasing factor production, which secondarily increase the production of pro-inflammatory cytokines (28,32,35). Figure I.1.4 summarizes the putative factors and mechanisms involved in the pathophysiology of depression.

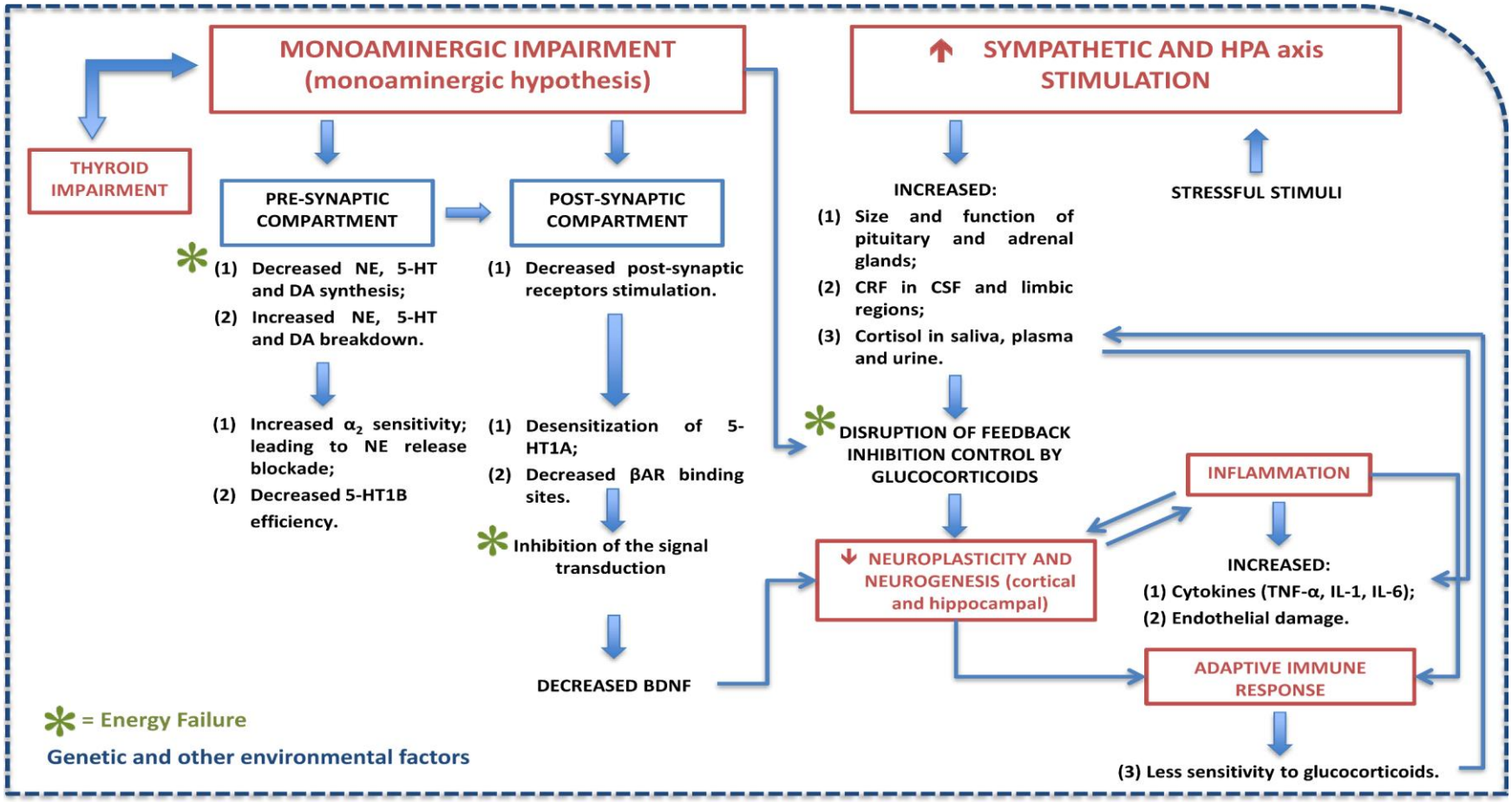


Figure I.1.4 Summary scheme of the putative factors and mechanisms involved in the pathophysiology of depression [adapted from (35)].

↓, decreased; ↑, increased; 5-HT, serotonin; 5-HT1A, 1A serotonin receptor; 5-HT1B, 1B serotonin receptor; βAR, β-adrenergic receptor; BDNF, brain-derived neurotrophic factor; CRF, corticotropin-releasing factor; CSF, cerebrospinal fluid; DA, dopamine; HPA, hypothalamus-pituitary-adrenal axis; IL, interleukin; NE, norepinephrine; TNF-α, tumour necrosis factor alpha.

I.2 PHARMACOLOGICAL TREATMENT

I.2 PHARMACOLOGICAL TREATMENT

I.2.1 GENERAL OVERVIEW

The treatment of depressive disorders is often based on a trial-error approach, where the treatments are tailored, as much as possible, to the patient and its clinical case and are guided by the clinical outcomes (efficacy *versus* adverse effects). Globally, the goals of the treatment of a depressive episode are to eliminate or reduce the symptoms, minimize adverse effects, ensure compliance with the therapeutic regimen, facilitate a return to a premorbid level of functioning and prevent further episodes of depression. The treatment of depression is nowadays divided in non-pharmacological (e.g. psychotherapy and electroconvulsive therapy) and pharmacological options (e.g. antidepressant and antipsychotic drugs). These are managed according to several individual factors, namely patient's clinical status and preference, previous experience, psychiatric and non-psychiatric co-morbidities, co-medication and potential for drug-drug interactions, but particularly, according to the severity of the depressive episode (mild, moderate or severe). On this matter, a number of national and international psychiatric and professional bodies have produced clinical practice guidelines (52,53,62,54–61). Despite some divergences in the threshold for initiating the pharmacological treatment between guidelines, psychotherapy and psychosocial interventions have been the main recommended approaches for the treatment of mild depressive episodes, with the pharmacotherapy being essentially advised for moderate to severe depressive episodes alongside with psychotherapy (56,63,64).

Regarding to pharmacotherapy of depression, antidepressant drugs have been considered as the gold-standard and, at this level, SSRIs have been the first-line option in relation to other classes of antidepressant drugs, namely SNRIs, mirtazapine or bupropion. Recommendations progressed from first-, second- to third-line drug treatments, with a tendency to broaden the antidepressant regimen from SSRIs (and SNRIs, mirtazapine, bupropion) to TCAs and monoamine oxidase inhibitors (MAOIs), with the latter ones recommended by some guidelines as being used earlier in cases of severe depression (56,63,64). Indeed, there is consensus on the first-line drug treatment recommendations amongst guidelines; on the other hand, the second- and third-line

recommendations and, specifically, the augmentation and combined antidepressant strategies are considerably variable. The use of combined antidepressant drugs, typically with different modes of action, has varied widely once there is little evidence to support any combination. Among the most used combinations of antidepressant drugs are: mirtazapine plus SSRI, mirtazapine plus venlafaxine (VEN) or mirtazapine plus TCA, SSRI plus TCA, TCA plus MAOI and bupropion or buspirone plus another antidepressant drug (56). Similarly, the clinical benefit of the adjunctive use of thyroid hormones, anticonvulsants and psychostimulants with antidepressant drugs is not completely clear. Contrarily, the addition of an atypical antipsychotic drug (e.g. aripiprazole, quetiapine, risperidone or olanzapine) or lithium to the current antidepressant treatment has been recognised as an efficacious augmentation strategy, mostly for the treatment of refractory depression and depression with psychotic features (56,63).

Finally, antidepressant pharmacotherapy is introduced using a progressive dose up-titration strategy focused on the optimization of the clinical outcomes. The majority of the guidelines recommend that the antidepressant treatment should be maintained for at least 6-12 months, following symptoms remission. For those clinical cases with multiple episodes (three or more episodes) or chronic depression, the antidepressant treatment has been recommended for lifetime. The suspension of the antidepressant treatment should also follow a progressive dose down-titration strategy (56,63).

I.2.2 ANTIDEPRESSANT DRUGS

The antidepressant drugs are well-known and widely used today not only for the treatment of depressive disorders, but also for the treatment of other psychiatric (e.g. generalized anxiety disorder, panic disorder, obsessive-compulsive disorder, posttraumatic stress disorder, and eating disorders) and non-psychiatric conditions (e.g. neuropathic pain, fibromyalgia, migraine, overactive bladder syndrome, irritable bowel syndrome and smoking cessation) (65). They have been classified in different classes, using different criteria and classification systems, such as classification based on their mechanism of action and/or chemical structure; however, no consensually accepted classification system exists. Despite this, the main antidepressant drugs currently available can be overall classified as: reuptake inhibitors, which increase the synaptic

concentration of monoamines by inhibiting their synaptic reuptake; MAOIs, which act to reduce the metabolic degradation of monoamines; and monoamine receptor modulators, which facilitate the monoamine neurotransmission. Depending on the class, they present particular characteristics in terms of monoamines and receptors selectivity (10,63,66,67). Table I.2.1 summarizes the main classes of antidepressant drugs currently available.

Table I.2.1 Main classes of antidepressant drugs currently available (10,63,66,67).

Class	Antidepressant drugs
Selective serotonin reuptake inhibitors (SSRIs)	Citalopram
	Escitalopram
	Fluoxetine (FLU)
	Fluvoxamine
	Paroxetine (PAR)
	Sertraline
Serotonin norepinephrine reuptake inhibitors (SNRIs)	Desvenlafaxine
	Duloxetine
	Levomilnacipran
	Milnacipran
	Venlafaxine (VEN)
Tricyclic antidepressants (TCAs)	Amitriptyline
	Clomipramine
	Desipramine
	Doxepin
	Imipramine
	Nortriptyline
Monoamine oxidase inhibitors (MAOIs)	Moclobemide
	Phenelzine
	Selegiline
	Tranylcypromine
Tetracyclics	Maprotiline
	Mirtazapine
Aminoketone	Bupropion
Triazolopyridines	Nefazodone
	Trazodone

Before 1950, there were no antidepressant drugs as we know today and depressive disorders were treated by amphetamine stimulants or electroconvulsive therapy (10,63). The first generation of antidepressant drugs were MAOIs and TCAs, which were discovered by chance in the late 1950s. The first antidepressant compound to be

discovered was the antitubercular agent iproniazid, a derivative of isoniazid, which proved to have powerful mood-enhancing properties, becoming the forerunner of the MAOIs (66,67). In turn, while searching for "chlorpromazine-like" compounds to treat schizophrenia, Roland Kuhn recognized the antidepressant properties of imipramine, that would be the first TCA (63,66,67). Other antidepressant drugs, from the same and different pharmacological classes, were being developed over the years, particularly to improve the safety profile of the treatment of depression (Table II.2.1) (63,66,67). As previously mentioned, the understanding of the mechanisms of action of these different classes of antidepressant drugs constituted the main evidence for the postulation of the monoaminergic hypothesis (28,30,32,34,66,67). TCAs overall increase the concentrations of norepinephrine and serotonin within the neuronal synapse by non-selectively or selectively inhibiting its reuptake. The selectivity for norepinephrine or serotonin transporters depends on the drug, but most TCAs are more selective for the norepinephrine transporter than for the serotonin transporter. In turn, MAOIs increase the concentrations of norepinephrine, serotonin and dopamine by inhibiting its metabolism, also through non-selectively or selectively inhibition of monoamine oxidase enzymes (MAO-A and MAO-B) (10,63,66,67). However, these drugs have reported limited tolerability and significant mechanism-based adverse effects. Specifically, MAOIs showed a high potential to cause life-threatening hypertensive reactions ("cheese reaction") by decreasing the intestinal and hepatic degradation of dietary sources of tyramine. The inhibition of monoamine oxidase enzymes allows excessive amounts of dietary tyramine, a sympathomimetic vasoconstrictor, resulting in increased blood pressure. Furthermore, the inhibition of monoamine oxidases was also involved in drug-drug interactions with monoamine drugs (10,63,66,67). Thus, the potential for food-drug and drug-drug interactions of MAOIs limited its therapeutic use and TCAs became the gold-standard for the treatment of depressive disorders between 1960 and 1980 (63,66). Nonetheless, TCAs were also related to tolerability issues, namely anticholinergic, anti-histaminergic and cardiotoxic adverse effects, due to their additional affinity for muscarinic, histamine and adrenergic receptors, as well as for cardiac and central nervous system sodium channels. This fact motivated the research of new antidepressant drugs with an improved reuptake selectivity and tolerability, namely SSRIs and new SNRIs (63,66,67). With the introduction of these newer antidepressant drugs between 1980s and 1990s, the use of TCAs started

to reduce and SSRIs became the first-line antidepressant drugs for all depressive disorders, due to their advantageous pharmacokinetics and safety profile (63,66,67). Nonetheless, this class of antidepressant drugs is not devoid of adverse effects or other therapeutic concerns, including delayed onset of therapeutic effects or therapeutic failure. Consequently, in the last years, drug research and development has been centred on new non-monoaminergic antidepressants (e.g. glutamatergic-based drugs) (63,68–70). Despite promising results, the introduction of new antidepressant drugs in the clinical practice has not been successful and SSRIs remain nowadays the first-line drug treatment for depressive disorders (56,68). In fact, consistent evidence has shown that SSRIs, as a group, are better tolerated than TCAs and display a similar efficacy profile for the treatment of depressive disorders. While the efficacy of the different classes of antidepressant drugs has overall been assumed as equivalent, differences in tolerability and safety profiles are well-accepted and have been responsible for the differences in its clinical use (63,66,67,71). However, there is no unequivocal evidence to support clinically significant differences in efficacy and tolerability among the various newer antidepressant agents, namely SSRIs and SNRIs, and literature is controversial at this level (71). Noteworthy, the long-term use of SSRIs and SNRIs is likely to yield important adverse effects, as summarized in the Table I.2.2 below.

Table I.2.2 Main adverse events related to the use of newer antidepressant drugs (selective serotonin reuptake inhibitors and serotonin-norepinephrine reuptake inhibitors) (71).

1.	Gastrointestinal (nausea, vomiting, gastrointestinal bleeding)
2.	Hepatotoxicity and hypersensitivity reactions
3.	Weight gain and metabolic disturbances
4.	Cardiovascular (QT interval prolongation, hypertension and orthostatic hypotension)
5.	Genitourinary (urinary retention and incontinence)
6.	Sexual dysfunction
7.	Hyponatremia
8.	Osteoporosis and fractures
9.	Bleeding
10.	Central nervous system (extrapyramidal effects, serotonin syndrome, headache and stroke)
11.	Sweating
12.	Sleep disturbances
13.	Affective (apathy, switching into hypomania or mania, paradoxical effects)
14.	Suicidality
15.	Discontinuation syndromes
16.	Ophthalmic (glaucoma and cataract)
17.	Hyperprolactinemia

Concluding, we may state that SSRIs and SNRIs are currently the most clinically relevant antidepressant drugs for the treatment of depressive disorders and, because of that, the present work was focused on drugs belonging to these two pharmacological classes. The following sections will provide a global overview about the clinical pharmacology of SSRIs and SNRIs, focusing on the antidepressant drugs in study, fluoxetine (FLU), paroxetine (PAR) and VEN.

I.2.2.1 SELECTIVE SEROTONIN REUPTAKE INHIBITORS

Historically, the SSRIs were the first class of psychotropic drugs to be rationally designed. Earlier, the serendipitously discovery of the TCAs provided the first evidence that the central serotonin agonism, by means of the inhibition of serotonin reuptake,

could be a way of producing antidepressant response. The knowledge of this important therapeutic principle opened the door for a rational process of drug research focused on agents that could selectively inhibit the reuptake of serotonin and minimise the undesired effects, such as the cardiovascular toxicity and anticholinergic properties of the TCAs (65,72–74). The first approved SSRI was zimelidine (1982), which was withdrawn from the market due to Guillain-Barré reactions. Later, five other SSRIs were released on the market, including fluvoxamine (1983), FLU (1987), citalopram (1989), PAR (1991), sertraline (1990) and escitalopram (2002) (66,67). Figure I.2.1 presents the chemical structures of the SSRIs drugs currently approved for the treatment of depression.

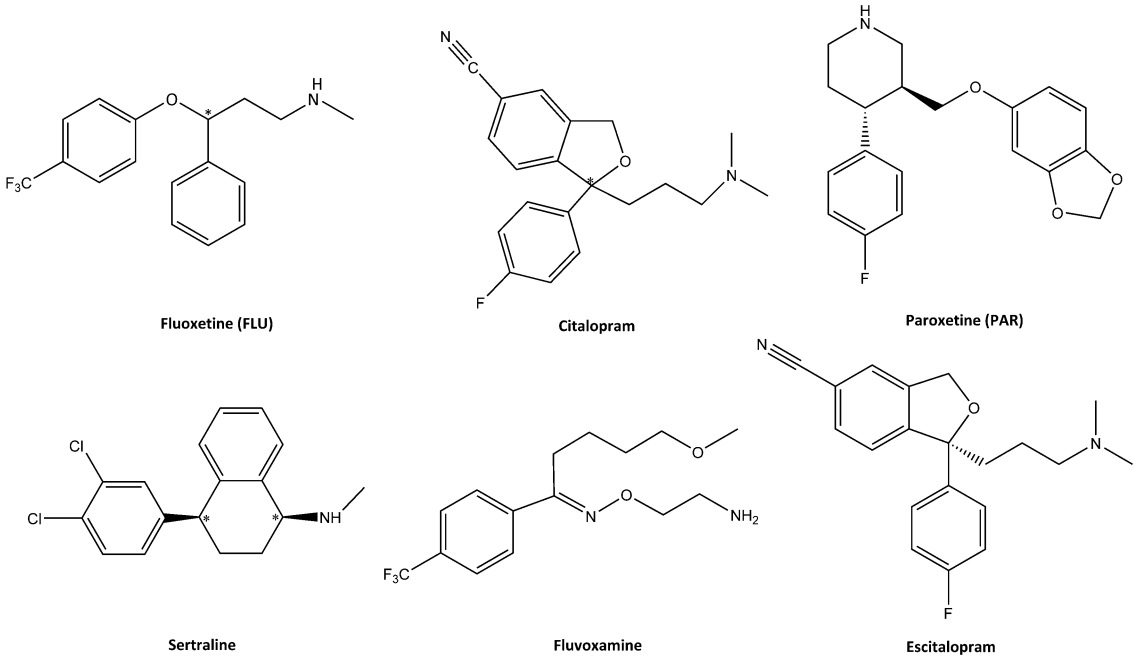


Figure I.2.1 Chemical structures of the selective serotonin reuptake inhibitors drugs currently approved for the treatment of depression (* - indicates a chiral centre) (75,76).

Despite the large differences in their chemical structures (Figure I.2.1), all SSRIs share the same mechanism of action. SSRIs act on the pre-synaptic SERT, by selectively inhibiting its functional activity. SSRIs are 20-1500-fold more selective for inhibiting the reuptake of serotonin over norepinephrine and have minimal binding affinity for other post-synaptic receptors, such as adrenergic α_1, α_2, and β, histamine H_1, muscarinic, and dopamine D_2 receptors. SSRIs also do not pre-synaptically stimulate the release of serotonin or norepinephrine and have weak or no direct pharmacological action at post-

synaptic serotonin receptors. Therefore, the serotoninergic effects produced by SSRIs is the result of increased concentrations of serotonin in the synaptic cleft via reuptake inhibition rather than direct post-synaptic stimulation. All the SSRIs have been considered as equally effective at doses adjusted to the patient. Long-term administration of SSRIs causes downregulation of pre- and post-synaptic receptors, a reduction in the amount of serotonin produced in the central nervous system and a reduction in the number of SERTs expressed. These compensatory responses at receptors and transporters take at least two weeks to occur and have been linked to the antidepressant effects of SSRIs. Thus, this delay in such compensatory responses may explain, at least in part, the delayed onset of action of SSRIs in the treatment of depression (63,65,72–74,77).

Meaningful differences between the individual SSRIs are largely related to their pharmacokinetics, metabolism, inhibition effects on the cytochrome P450 (CYP) isoenzymes and potential for drug–drug interactions. Table I.2.3 summarizes the pharmacokinetic properties of the main SSRIs currently available.

Table I.2.3 Pharmacokinetic properties of the selective serotonin reuptake inhibitors.

Parameters	Fluoxetine	Paroxetine	Sertraline	Fluvoxamine	Citalopram	Escitalopram	Refs
Usual dose range (mg/day)	20-80	20-60	25-200	100-300	20-60	10-20	(78)
Oral bioavailability (%)	60-80	50	20-36	53	80	80	(79–85)
Protein binding (%)	95	95	96-98	77-80	80	56	(79–85)
Volume of distribution (L/kg)	20-45	25	17	25	12	12	(79–85)
Half-life	acute administration: 1-3 days-, chronic administration: 4-6 days; norfluoxetine: 4-16 days	21-24 h	26 h (desmethylsertraline: 62-104 h)	15.6 h	35 h	27-32 h	(79–85)
CYP isoenzymes involved in metabolism	**2C9**, 2C19, **2D6**, 3A4, 3A5	1A2, 2C19, **2D6**, 3A4, 3A5	**2B6,** 2C19, 2C9, 2D6, 3A4	**2D6**, 1A2	**2C19**, 2D6, **3A4**	**2C19**, 2D6, **3A4**	(86)
Major active metabolite(s)	Norfluoxetine	None	Desmethylsertraline (< activity than its parent compound)	None	Desmethylcitalopram (< activity than its parent compound)	*S*-desmethylcitalopram and *S*-didesmethylcitalopram	(79–85)

Table I.2.3 Pharmacokinetic properties of the selective serotonin reuptake inhibitors.

Parameters	Fluoxetine	Paroxetine	Sertraline	Fluvoxamine	Citalopram	Escitalopram	Refs
P-gp substrate	Not clear	Yes	No	Yes	Yes	Yes	(87)
T_{max} (h)	6-8	2-8	4-8	3-8	4	4	(79–85)
Excretion	Urine (60 %) Faeces minor	Renal (64 %) Faeces (36 %)	Urine (51–60 %) Faeces (24–32 %)	Urine (94 %)	Faeces (80–90 %) Urine (< 5 %)	Urine major	(79–85)
CYP Inhibitor							(63)
1A2	+	+	+	+++	+	0	
2C9	++	+	+	++	0	0	
2C19	+ to ++	+	+ to ++	+++	0	0	
2D6	+++	+++	+	+	0	+	
3A	+	+	+	+	0	0	
2B6	+	+++	+	+	0	0	
Time to steady-state (days)	28-35	7-14	7-10	10	7	7–10	(79–85)
Linear pharmacokinetics	No	No	Yes	No	Yes	Yes	(79–85)
Recommended therapeutic concentration (ng/mL)	120–500 (fluoxetine + norfluoxetine)	30–120	10–150	60–230	50–110	15–80	(70,88)

0, minimal or zero inhibition; +, mild inhibition; ++, moderate inhibition; +++, strong inhibition; CYP, Cytochrome P450; P-gp, P-glycoprotein; T_{max}, time to reach the maximum concentration. Enzyme(s) in bold represent(s) the major metabolic route(s).

Overall, SSRIs are highly lipophilic drugs, well absorbed and extensively distributed after oral administration. They highly bind to plasma proteins and yet have high volumes of distribution (Table I.2.3) (70,77,89). Moreover, SSRIs have been found as substrates of P-glycoprotein (P-gp), with exception of sertraline and FLU. However, for FLU, literature is not conclusive and the potential involvement of this efflux transporter on its pharmacokinetics remains unclear (87). In terms of elimination, SSRIs are extensively metabolised by CYP isoenzymes (mainly by CYP2C9, CYP2C19 and CYP2D6) to pharmacologically active *N*-demethylated or non-active metabolites, which are then excreted in urine and faeces (Table I.2.3). The involvement of P-gp, CYP2C9, CYP2C19 and CYP2D6 in the pharmacokinetics of the SSRIs is noteworthy, once these proteins have been acknowledged as relevant pharmacokinetic-related sources of inter-individual variability, due to genetic and non-genetic factors (e.g. co-medication). On the other hand, SSRIs exert different inhibitory effects on the CYP isoenzymes and, hence, may interfere with the metabolism of other co-administered CYP metabolized drugs (Table I.2.3). Among the SSRIs, FLU, PAR and fluvoxamine are those with higher potential for drug-drug interactions by inhibition of the CYP isoenzymes, particularly the CYP2C9, CYP2C19 and CYP2D6. Of note, FLU, PAR and fluvoxamine inhibit their own metabolism (63,70,77,89). The half-life of the SSRIs depends on the individual drug, but it tends to be prolonged and is consistent with once-daily administration. Consequently, the time to reach the steady-state conditions is usually long and the washout period before switching to another serotonergic agent is relatively long (at least 5 weeks for FLU and 2 weeks for the remaining SSRIs) (70,77,89). Importantly, sertraline, citalopram and escitalopram exhibit linear pharmacokinetics, whereas fluvoxamine, FLU, and PAR exhibit non-linear pharmacokinetics, due to the inhibition of their own metabolism (63,77,89–91).

Overall, SSRIs as a class have similar efficacy to TCAs and MAOIs for depressive disorders and a lower tendency to cause severe cardiovascular effects (78). Escitalopram and PAR were recently found among the most efficacious antidepressant drugs for the treatment of adult patients with unipolar major depressive disorder and FLU and fluvoxamine among the least ones. In turn, citalopram, escitalopram, FLU and sertraline were found among the antidepressant drugs with better tolerability profile and fluvoxamine is among those with the worst tolerability profile. Globally, escitalopram, PAR

and sertraline were among the drugs with a higher response and lower dropout rate for the treatment of adult patients with unipolar major depressive disorder (92).

Regarding the SSRIs-related adverse effects (Table I.2.2), the majority of them are due to serotoninergic effects and, therefore, are dose-related effects and may usually be alleviated by dosing adjustments. During long-term treatment with SSRIs, the most common and troublesome adverse effects are weight gain, sleep disturbance and sexual dysfunction. Sexual dysfunction is by far the most common SSRIs-related adverse effect and it has been explained by the stimulation of 5-HT_2 and 5-HT_3 receptors. Delayed ejaculation, anorgasmia, and decreased libido may occur in up to 60 % of patients treated with SSRIs. In turn, sleep disturbances, either insomnia or somnolence, have been reported in approximately 25 % of patients taking SSRIs. Importantly, like all other antidepressants, SSRIs may cause an increase in suicide ideation and in the rate of suicide attempts, although this is not a consensual matter, because these symptoms may also be a consequence of the psychiatric disorder (63,71,77,93,94). SSRIs have also been associated with an increased risk of bleeding, in particular in the upper gastrointestinal tract, essentially when combined with drugs interfering with haemostasis (e.g. nonsteroidal anti-inflammatory drugs, oral anticoagulants and antiplatelet drugs). Such increased risk of bleeding has been described as a consequence of both pharmacokinetic and pharmacodynamic interactions. First, SSRIs and oral anticoagulants (e.g. warfarin) are both highly bound to plasma proteins; thus, SSRIs may precipitate displacement interactions when co-administered with oral anticoagulants and indirectly potentiate the anticoagulant effect, increasing the bleeding risk. Then, some SSRIs, particularly fluvoxamine and FLU, may increase the risk of bleeding associated with warfarin through inhibition of the CYP2C9- mediated oxidative metabolism of the more biologically active enantiomer of warfarin (*S*-enantiomer). On the other hand, it has been stated that SSRIs may reduce platelet aggregation by depleting platelet serotonin levels (65,71,95). Also, although constitutes a rare condition, serotonin syndrome should be highlighted due to its serious clinical picture. This is a rare adverse effect of SSRIs and results from a serotoninergic hyperstimulation, commonly caused by the co-administration of serotoninergic agents (including MAOIs, TCAs, SNRIs, L-tryptophan, triptans, tramadol, linezolid, lithium and St. John's Wort), which is manifested as alterations in metal status, restlessness, myoclonus, hyperreflexia, diaphoresis and tremor (63,71,77,93,94). Finally,

the abrupt discontinuation of the SSRIs may lead to an abstinence syndrome, characterized by somatic and psychological symptoms such as dizziness, nausea, weakness, insomnia, anxiety, irritability, and headache. It is therefore advised a gradual dose down-titration during a period of at least one to two weeks (63,71,77,93,94).

I.2.2.1.1 FLUOXETINE

FLU is a 3-phenoxy-3-phenylpropylamine (Figure I.2.1) and it is considered the prototype drug of the SSRIs class. In 1994, it was the second best-selling drug worldwide and today remains as one of the most prescribed drugs (66,67,78). FLU is approved for the treatment of major depressive disorder, moderate to severe bulimia nervosa, obsessive-compulsive disorder, premenstrual dysphoric disorder, panic disorder and for treatment-resistant or bipolar depression in combination with olanzapine (79,80,96). FLU is commercialised as a racemic mixture of the *R*- and *S*-enantiomers and it is usually administered once daily at typical doses of 20-80 mg (78,80).

I.2.2.1.1.1 PHARMACOKINETICS

FLU is well absorbed from the gastrointestinal tract following oral administration. However, its systemic bioavailability is reduced because of first-pass metabolism in the liver. Oral bioavailability is estimated to be at least 60-80 % and maximum plasma concentrations occur within 6-8 hours (79,80). The oral bioavailability is not affected by food intake. FLU extensively binds to plasma proteins (about 95 %) and it is widely distributed to the tissues (Table I.2.3) (79,80,86).

Regarding metabolism, FLU is extensively metabolized in the liver to its main active metabolite norfluoxetine (NFLU) and to other non-active metabolites. NFLU is formed via *N*-demethylation of FLU. Figure I.2.2 shows the metabolic pathway of FLU (79,80,86). Patients under chronic treatment with FLU at the usual doses have comparable FLU and NFLU plasma levels and, therefore, it is usually accepted that the metabolite NFLU significantly contributes to the therapeutic effect of FLU (78). FLU and NFLU both undergo phase II glucuronidation reactions. These forms are then predominantly excreted by urine, with less than 10 % excreted as unchanged FLU or FLU glucuronide (79,80,86). While

the interaction of FLU with P-gp is unclear, it is known that NFLU is not a substrate of P-gp (87).

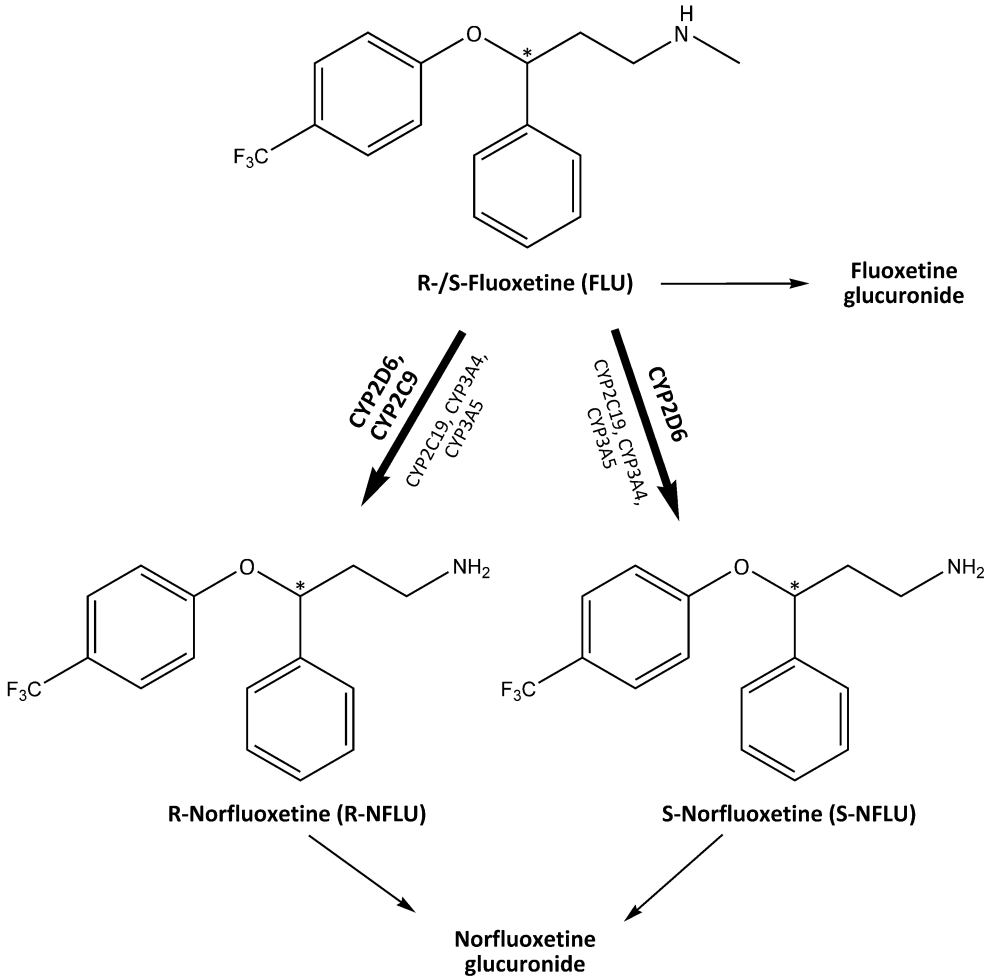


Figure I.2.2 Metabolic pathway of fluoxetine in humans (86). The bold arrow indicates the major metabolic pathway. The main metabolizing enzyme(s) of each pathway is/are indicated above the arrows, at superior size. CYP, cytochrome P450.

FLU is a chiral compound, but once both enantiomers [*S*-fluoxetine (*S*-FLU) and *R*-fluoxetine (*R*-FLU)] have similar serotonin reuptake properties, therapy is carried out using the racemate. However, *S*-norfluoxetine (*S*-NFLU) is more potent than *R*-FLU and the pharmacokinetics of FLU and NFLU exhibits marked stereoselectivity towards the *S*-enantiomers (78,86,97,98). In steady-state conditions, the plasma concentrations of *S*-FLU and *S*-NFLU were found to be two times greater than the plasma concentrations of the respective *R*-enantiomers [*R*-FLU and *R*-norfluoxetine (*R*-NFLU)] (86,97,98). Evidence from *in vitro* and *in vivo* studies indicates the involvement of the CYP2D6, CYP2C19,

CYP2C9, CYP3A4, and CYP3A5 in the biotransformation of *R*- and *S*-FLU to their *N*-desmethyl metabolites (*R*-NFLU and *S*-NFLU]. CYP2D6 and CYP2C9 are the major FLU metabolizing isoenzymes; CYP2D6 and CYP2C9 seem to preferentially catalyse *R*-FLU demethylation, whereas the formation of *S*-NFLU is highly dependent of the CYP2D6 (Figure I.2.2) (86). In this scope, considerable inter-individual variability has been found at the level of metabolism and pharmacokinetics of FLU with potential to affect the clinical outcomes, especially related to genetic polymorphisms of CYP2D6 and CYP2C9 (78,86).

At the same time, FLU and NFLU are strong inhibitors of CYP2D6, inhibiting their own metabolism. Indeed, FLU may induce the phenoconversion of CYP2D6 extensive metabolizers (EMs) into poor metabolizers (PMs) (63,86). Additionally, FLU has demonstrated inhibitory potency toward other CYP isoenzymes, namely CYP1A2, CYP2C9, CYP2C19, CYP3A and CYP2B6 (Table I.2.2) (63). This autoinhibition of its own metabolism explains the long half-life of FLU and NFLU and its non-linear pharmacokinetics. On the other hand, all these CYP isoenzymes are involved in the metabolism of numerous drugs; therefore, FLU has a high potential to alter the metabolism and pharmacokinetics of co-administered drugs metabolised by these CYP isoenzymes and to trigger drug-drug interactions (86). Clinically relevant pharmacokinetic-based interactions involving FLU, as precipitant or victim drug, have been reported (74,78,80,95). For instance, plasma and brain concentrations, as well as the therapeutic efficacy of both FLU and TCAs can be increased by their simultaneous administration. Other clinically relevant examples of the CYP2D6 inhibition mediated by FLU are the increased plasma concentrations of clozapine, olanzapine, lithium and diazepam when co-administered with FLU (78).

Lastly, in conditions of hepatic impairment, FLU and NFLU half-lives can be increased to 7 and 12 days, respectively; thus, a lower or less frequent dose should be considered. In turn, the pharmacokinetics of FLU does not appear to be substantially changed in patients with renal impairment, neither in healthy elderly. Nonetheless, because of its relatively long half-life and non-linear pharmacokinetics, the possibility of pharmacokinetic changes in elderly exist, particularly in those in a comorbid and/or polymedicated state.

I.2.2.1.1.2 PHARMACODYNAMICS

FLU shares the pharmacodynamic profile of the SSRIs, being a potent and selective inhibitor of serotonin reuptake in the central nervous system. As previously mentioned, although *S*- and *R*- FLU are approximately equivalent in their ability to inhibit serotonin reuptake, their metabolites, *S*- and *R*-NFLU, are not. *S*-NFLU has about 20-times higher potency in blocking serotonin reuptake than the *R*-NFLU (63,86).

As a SSRI drug, FLU has been described as a drug with clinical efficacy similar to TCAs for depressive disorders, but with less cardiovascular and anticholinergic adverse effects (78,80). Indeed, FLU was recently found as one of the antidepressant drugs with better tolerability; however, it was also found as one of the least efficacious (92). The adverse effects profile of FLU is common to the SSRIs. The most common adverse effects are nausea, insomnia, nervousness and somnolence, which tend to disappear with the treatment continuation; on the contrary, restlessness, tension, agitation, and sleep disturbance can develop after long periods of chronic FLU use (78,80). Moreover, anorexia and weight loss have been identified as adverse effects particularly related to FLU (78). FLU is also one of the SSRIs with a higher risk of platelet dysfunction and bleeding. However, it is one of the SSRIs with lower overdose-related mortality rate (71).

I.2.2.1.2 PAROXETINE

PAR is a chemical analogue of FLU (phenoxyphenylalkylamine) (Figure I.2.1) approved for the treatment of major depressive disorder, panic disorder with or without agoraphobia, obsessive-compulsive disorder, social anxiety disorder (social phobia), generalized anxiety disorder, post-traumatic stress disorder and premenstrual dysphoric disorder. Among the SSRIs, PAR has the most evidence supporting its use for anxiety-related disorders, including depression associated with anxiety, and for depression resistant to other antidepressants (79,81). Usual doses of PAR are between 20 to 60 mg, once daily (81).

I.2.2.1.2.1 PHARMACOKINETICS

PAR is well absorbed after oral dosing and, similarly to FLU, undergoes first-pass metabolism, which decreases its absolute bioavailability to about 50 %. It is extensively distributed into tissues, with only 1 % remaining in the plasma, and it is also highly bound to the plasma proteins (95 %) (79,81). PAR has been consensually described as a substrate of P-gp (Table I.2.3) (87).

In terms of metabolism, PAR is extensively metabolized to inactive metabolites. The main metabolites are polar and conjugated products of oxidation and methylation (glucuronic acid and sulphate conjugates), which are quickly eliminated (78,79,81). Figure I.2.3 displays the putative metabolic pathway of PAR.

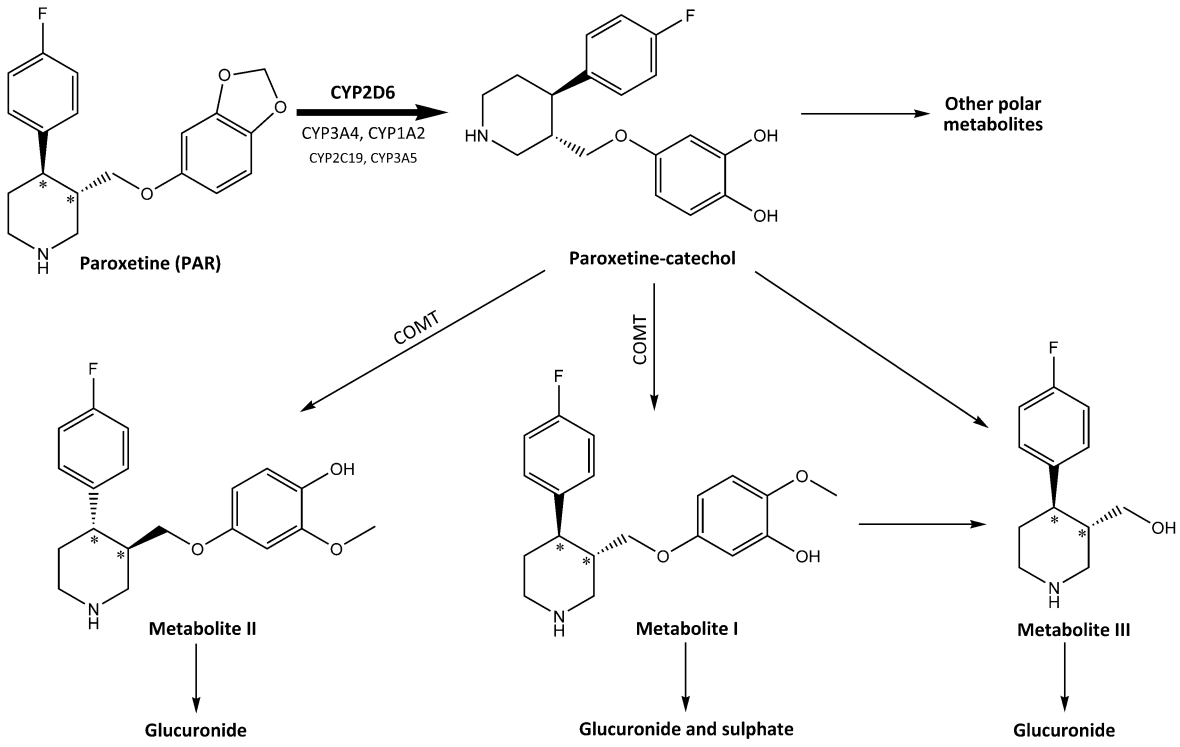


Figure I.2.3 Metabolic pathway of paroxetine in humans (86,99). The bold arrow indicates the major metabolic pathway. The main metabolizing enzyme(s) of each pathway is/are indicated above the arrows, at superior size. CYP, cytochrome P450; COMT, catechol-*O*-methyltransferase.

PAR is firstly metabolised to a catechol intermediate (PAR catechol), mainly by CYP2D6. *In vitro* studies indicate a minor involvement of CYP1A2, CYP2C19 and CYP3A4/5 in the PAR catechol formation. Data from population-based simulations suggested that

CYP3A4 and CYP1A2 are most likely to be involved in PAR metabolism in subjects with impaired CYP2D6 activity (CYP2D6 PMs) (86). PAR catechol is then metabolised to other metabolites, particularly by catechol-*O*-methyltransferase (COMT), which are subsequently conjugated with glucuronide and sulphate and excreted both in urine and in faeces (86). Urinary excretion of unchanged PAR is generally less than 2 % of dose whilst that of metabolites is about 64 %. About 36 % of the dose is excreted in faeces, probably via the bile, of which unchanged PAR represents less than 1 % of the dose. Thus, PAR is eliminated almost entirely by metabolism (79,81). It has been found that both renal and hepatic impairment, as well as age, have a big impact on the drug metabolism and excretion. Increased plasma concentrations of PAR have been observed in elderly, as well as in subjects with severe renal impairment and in those with hepatic impairment (78,81).

Like FLU, PAR inhibits its own metabolism, mainly via inhibition of CYP2D6 (Table I.2.3) (86). Beyond inhibiting its own metabolism, PAR also has potential to inhibit the metabolism of several drugs metabolised by CYP2D6. For instance, PAR was found to inhibit the metabolism of risperidone (causing increased weight gain), mirtazapine (increasing mirtazapine concentrations and the incidence of adverse effects) and TCAs (increasing TCA concentrations, but without apparent increase in adverse effects) (78). Additionally, the inhibition of CYP2D6 by PAR may also lead to increased plasma concentrations of other drugs, such as phenothiazine neuroleptics (e.g. perphenazine and thioridazine), certain type 1c antiarrhythmics (e.g. propafenone and flecainide) and metoprolol. Use of PAR and metoprolol when given in heart failure is contra-indicated, due to the narrow therapeutic index of metoprolol in this indication (81). Also, PAR should not be used in combination with thioridazine, once PAR may elevate the plasma concentrations of thioridazine and lead to QTc interval prolongation with associated serious ventricular arrhythmia, such as *torsades de pointes* and sudden death. Another example is procyclidine. Co-administration of PAR significantly increases the plasma concentrations of procyclidine, which may enhance its anticholinergic effects (81). Remarkably, it has been reported that PAR inhibits the activation of the anti-tumoral tamoxifen, which is a prodrug activated by CYP2D6 metabolism (78). Apart from CYP2D6, PAR also inhibits, to a lesser extent, CYP1A2, CYP2B6 CYP2C9, CYP2C19 and CYP3A4 (Table I.2.3) (78).

I.2.2.1.2.2 PHARMACODYNAMICS

PAR is probably the most selective of the SSRIs, being the most potent inhibitor of the reuptake of serotonin with weak anticholinergic properties. *In vitro* studies have shown that PAR has little affinity for α_1, α_2 and β-adrenoceptors, dopamine (D2), 5-HT1, 5-HT2 and histamine (H1) receptors (79,81). In the treatment of depressive disorders, PAR has been described as an antidepressant drug with comparable efficacy to the standard antidepressants (81). Recently, PAR was reported as one of the best antidepressant drugs for the treatment of unipolar major depressive disorder in adults, with a higher response and lower dropout rate in relation to other antidepressant drugs (92).

The most usual adverse effects of PAR are similar to those observed with FLU. However, PAR appears to be associated with an increased risk of extrapyramidal symptoms and withdrawal syndrome, especially if patients experience adverse effects in the early phase of the treatment. PAR has the greatest anticholinergic activity of the SSRIs and, for that reason, it may cause greater weight gain, sexual dysfunction, sedation, constipation and orthostatic hypotension (71,81). In fact, sexual dysfunction is a very common adverse effect of PAR (frequency ≥ 1/10) (81). In turn, although gastrointestinal bleeding is a very rare adverse effect of PAR (frequency < 1/10,000), such as FLU, also PAR is one of the SSRIs with high potential to cause platelet dysfunction (71). Furthermore, some studies have indicated a teratogenic potential of PAR, particularly an increased risk of congenital malformations and spontaneous abortion with exposure of PAR in the first pregnancy trimester. Thus, the use of PAR in pregnant women has not been recommended (71,78). Elevation of hepatic enzymes have also been rarely reported (81). Of note, PAR has been especially associated with withdrawal symptoms, once it is an antidepressant with a relatively short half-life (71).

I.2.2.2 SEROTONIN-NOREPINEPHRINE REUPTAKE INHIBITORS

The SNRIs are “dual action” antidepressant drugs, which act by inhibiting both serotonin and norepinephrine reuptake, with minimal or no pharmacological action on other receptors. VEN was the first SNRI introduced into the market (1993) for the treatment of major depressive disorder, being nowadays available in immediate (IR) and

extended-release formulations (XR). Since the introduction of VEN, other SNRI antidepressant drugs have been approved for this indication, including desvenlafaxine (*O*-desmethylvenlafaxine, ODV), duloxetine, milnacipran and levomilnacipran (66,100,101). Figure I.2.4 displays the chemical structures of these antidepressant drugs.

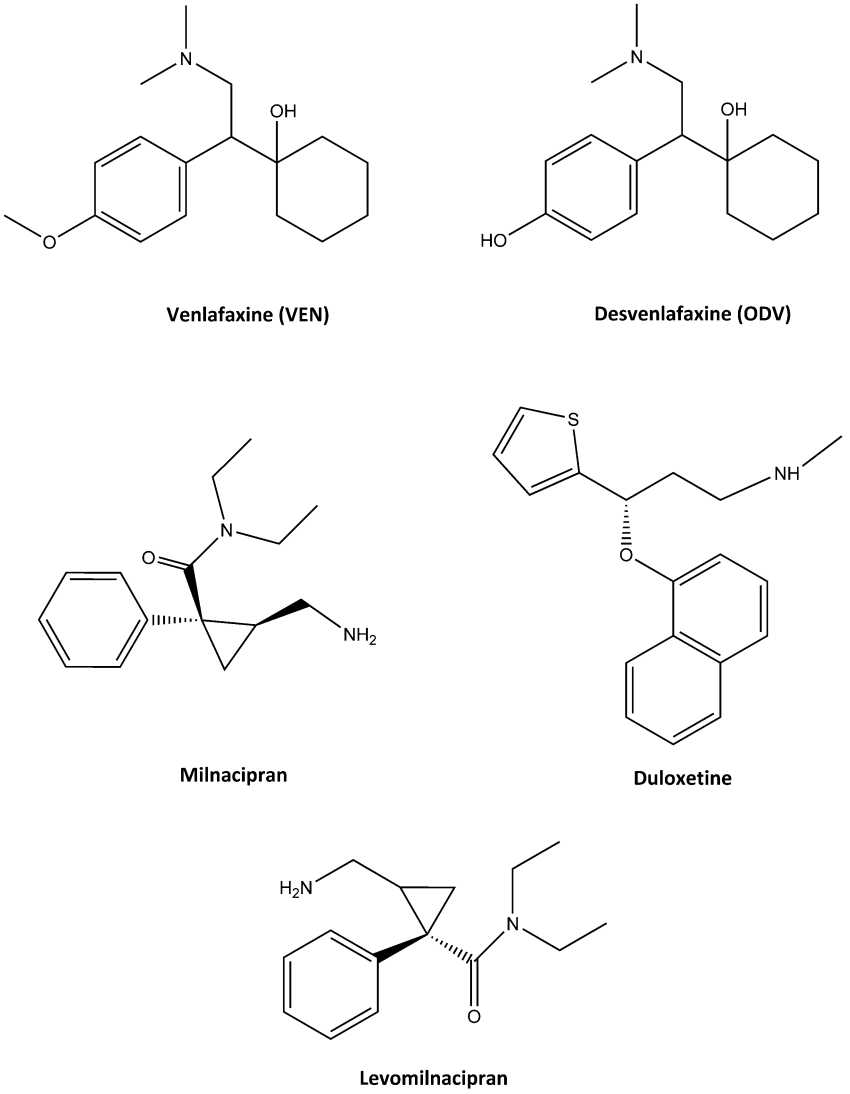


Figure I.2.4 Chemical structures of the serotonin-norepinephrine reuptake inhibitors drugs currently approved for the treatment of depression (75,76)

The main indication of the SNRIs is the treatment of depressive disorders, particularly in clinical cases with poor response or intolerability to the first-line SSRIs. In addition, SNRIs are used to treat panic disorder, generalized anxiety disorder, social anxiety disorder, obsessive-compulsive disorder, post-traumatic stress disorder, and body dysmorphic disorder. SNRIs have also demonstrated therapeutic effects for painful

diabetic peripheral neuropathy, fibromyalgia, menopausal hot flashes, vulvodynia, and urinary incontinence (101,102).

These drugs have repeatedly shown to be as efficacious as TCA drugs in treating major depressive disorders. In turn, several works have suggested that SNRIs may be more effective than SSRIs for the treatment of major depressive disorder (103–109). However, as previously addressed, not all studies support this conclusion and this point is not consensual in literature (66,71,100,101,110–112). On the other hand, well-recognised is that this class of antidepressant drugs tend to cause a broader array of adverse effects than the first-line SSRIs, including signs of noradrenergic activity (66,71,100,101).

Like for the SSRIs, differences between the individual SNRIs are largely related to their metabolism and pharmacokinetics, effects of inhibition on the CYP isoforms and potential for drug–drug interactions. Table I.2.4 summarizes the pharmacokinetic properties of the main SNRI drugs currently available.

Overall, SNRIs are well-absorbed drugs (101). In comparison with other types of antidepressants, such as the SSRIs and TCAs, the SNRIs have low plasma protein binding, relatively short half-lives and few-to-no active metabolites, suggesting a simpler pharmacology (Table I.2.4) (100). Among the five SNRIs, milnacipran and VEN IR are dosed twice daily, whereas the remaining ones, including venlafaxine XR, are dosed once daily (100). Only VEN is metabolised to a relevant active metabolite (ODV); ODV, duloxetine, milnacipran and levomilnacipran do not present known active metabolites (86,100). Similarly to the SSRIs, also CYP2C9, CYP2C19 and CYP2D6 are the most relevant SNRIs-metabolizing enzymes. P-gp is also involved in the pharmacokinetics of this class of antidepressant drugs (Table I.2.4). On the other hand, SNRIs have a low potential to precipitate pharmacokinetic-based drug interactions, by interfering with drug-metabolising CYP isoenzymes. Just duloxetine is a moderate CYP2D6 inhibitor; VEN, ODV, milnacipran and levomilnacipran do not potently or moderately inhibit CYP isoenzymes (Table I.2.4) (100,101).

Table I.2.4 Pharmacokinetic properties of the serotonin-norepinephrine reuptake inhibitors.

Parameters	Venlafaxine (VEN)	Desvenlafaxine (ODV)	Duloxetine	Milnacipran	Levomilnacipran	Refs
Usual dose range (mg/day)	75-375	50	60-120	25-200	40-120	(102)
Oral bioavailability (%)	40-45	80	32-80	85-90	92	(113–116)
Protein binding (%)	27	30	96	15-30	22	(113–116)
Volume of distribution (L/kg)	7.5 ± 3.7	3.4	1640 L	17	387 - 473 L	(113–116)
Half-life	5 ± 2 h	11 h	8-17 h	12 h	12 h	(113–116)
CYP enzymes involved in metabolism	1A2**, 2D6,** 2C9, 2C19, 3A4	**3A4, 2C19**, 2C9	1A2, 2D6	None (metabolism via glucuronidation)	**CYP3A4**, CYP2C8, 2C19, 2D6	(86)
Major active metabolite(s)	*O*-desmethylvenlafaxine (ODV/desvenlafaxine)	None	None	None	None	(113–116)
P-gp substrate	Yes	Yes	Unknown	Unknown	Yes	(87)

Table I.2.4 Pharmacokinetic properties of the serotonin-norepinephrine reuptake inhibitors.

Parameters	Venlafaxine (VEN)	Desvenlafaxine (ODV)	Duloxetine	Milnacipran	Levomilnacipran	Refs
T_{max} (h)	IR: 2 h (VEN) and 3 h (ODV) XR: 5.5 h (VEN) and 9 h (ODV)	7.5	6	2-4	6-8	(113–116)
Major excretion route	Urine	Urine	Urine	Urine	Urine	(113–116)
CYP Inhibitor						(63)
1A2	0	0	0	0	0	
2C9	0	0	0	0	0	
2C19	0	0	0	0	0	
2D6	0/+	+	++	0	0	
3A	0	0	0	+	0	
2B6	0	0	0	0	0	
Time to steady-state (days)	3	4-5	3-7	2-3	-	(113–116)

Table I.2.4 Pharmacokinetic properties of the serotonin-norepinephrine reuptake inhibitors.

Parameters	Venlafaxine (VEN)	Desvenlafaxine (ODV)	Duloxetine	Milnacipran	Levomilnacipran	Refs
Linear pharmacokinetics	Yes	Yes	Yes	Yes	Yes	(113–116)
Recommended therapeutic concentration (ng/mL)	100-400 (VEN + ODV)	100-400	30–120	100–150	80–120	(70,88)

0, minimal or zero inhibition; +, mild inhibition; ++, moderate inhibition; +++, strong inhibition; CYP, Cytochrome P450; IR, immediate release; P-gp, P-glycoprotein; T_{max}, time to the maximum concentration; XR, extended-release. Enzyme(s) in bold represent(s) the major metabolic route(s).

Regarding pharmacodynamics, the SNRIs vary in their affinity for the serotonin and norepinephrine transporter. VEN inhibits with higher potency the serotonin reuptake than the norepinephrine reuptake. Both duloxetine and ODV demonstrate less imbalance between the reuptake of serotonin and norepinephrine, but still retain greater potency for serotonin reuptake inhibition. In contrast, milnacipran exerts a relatively identical influence on the serotonin and norepinephrine reuptake inhibition, whereas levomilnacipran presents a reversed profile. Importantly, both VEN and duloxetine exhibit dose-related sequential effects on the reuptake inhibition, first affecting the serotoninergic system and then the noradrenergic system. This results in a sequential adverse effect profile, with the initial onset of serotonergic adverse effects followed by noradrenergic effects. While the status of ODV at this level is unclear, milnacipran and levomilnacipran simultaneously act on the serotonin and norepinephrine systems. Globally, the SNRIs have little or no effect upon dopaminergic, cholinergic, histaminergic and α_1-adrenergic receptors (100,101). Nonetheless, these drugs stimulate the norepinephrine receptors in the sympathetic nervous system, leading to a decrease in the parasympathetic effects. Despite the lack of direct effects on the cholinergic receptors, this may cause “pseudo-anticholinergic” adverse effects (e.g. constipation, dry mouth and urinary retention) (101). Apart from these effects, SNRIs and SSRIs overall share a common adverse effects profile, as described in the Table I.2.2 (71). Additionally, SNRIs have been more frequently associated than SSRIs to gastrointestinal and urinary adverse effects, sleep disturbances, increased pulse and blood pressure and agitation, due to the extra noradrenergic effects (71,101). In turn, SNRIs have also been associated with sexual dysfunction and risk of bleeding, although the association between the risk of bleeding and SNRIs is less compelling than with SSRIs (71). They are also contra-indicated in patients who received MAOIs in the previous two weeks and they should be used cautiously with other serotonergic drugs because of the risk of serotonin syndrome. Abstinence syndrome is a concern as well and patients should not abruptly discontinue SNRI antidepressants. This discontinuation syndrome is particularly common with VEN and relatively uncommon with milnacipran (101).

I.2.2.2.1 VENLAFAXINE

VEN is a hydroxycycloalkylphenylethylamine-derivative antidepressant drug (Figure I.2.4) approved for the treatment of depression and anxiety disorders (77,95,117–120). Presently, VEN is often used as an alternative drug for the treatment of SSRIs-resistant depression being, therefore, one of the antidepressant agents most commonly prescribed worldwide (111,119,121–125). In the treatment of depressive disorders, VEN is typically used at doses of 75-375 mg (Table I.2.4), administered twice-daily (IR formulation) or once-daily (XR formulation) (126).

I.2.2.2.1.1 PHARMACOKINETICS

VEN is rapidly and extensively absorbed from the gastrointestinal tract after oral administration. Food slightly delay the rate of absorption, but does not affect the absolute bioavailability (127,128). Mass balance studies in humans support that at least 92 % of VEN is absorbed following a single oral dose. Nevertheless, VEN has an absolute oral bioavailability of only 40-45 %, due to the extensive first-pass metabolism to the major metabolite ODV (118,119,127,129).

Since ODV is pharmacologically equivalent to the parent drug, no therapeutic consequences of this extensive pre-systemic metabolism are anticipated (130). Besides ODV, other minor metabolites [*N*-desmethylvenlafaxine (NDV), *N,O*-didesmethylvenlafaxine (DDV) and *N,N,O*-tridesmethylvenlafaxine (TDV)] are also formed by secondary metabolic pathways (127,131,132), which have been described as less active derivatives devoid of clinical relevance (133,134). A schematic overview of the known metabolic pathways for VEN in humans is presented in Figure I.2.5.

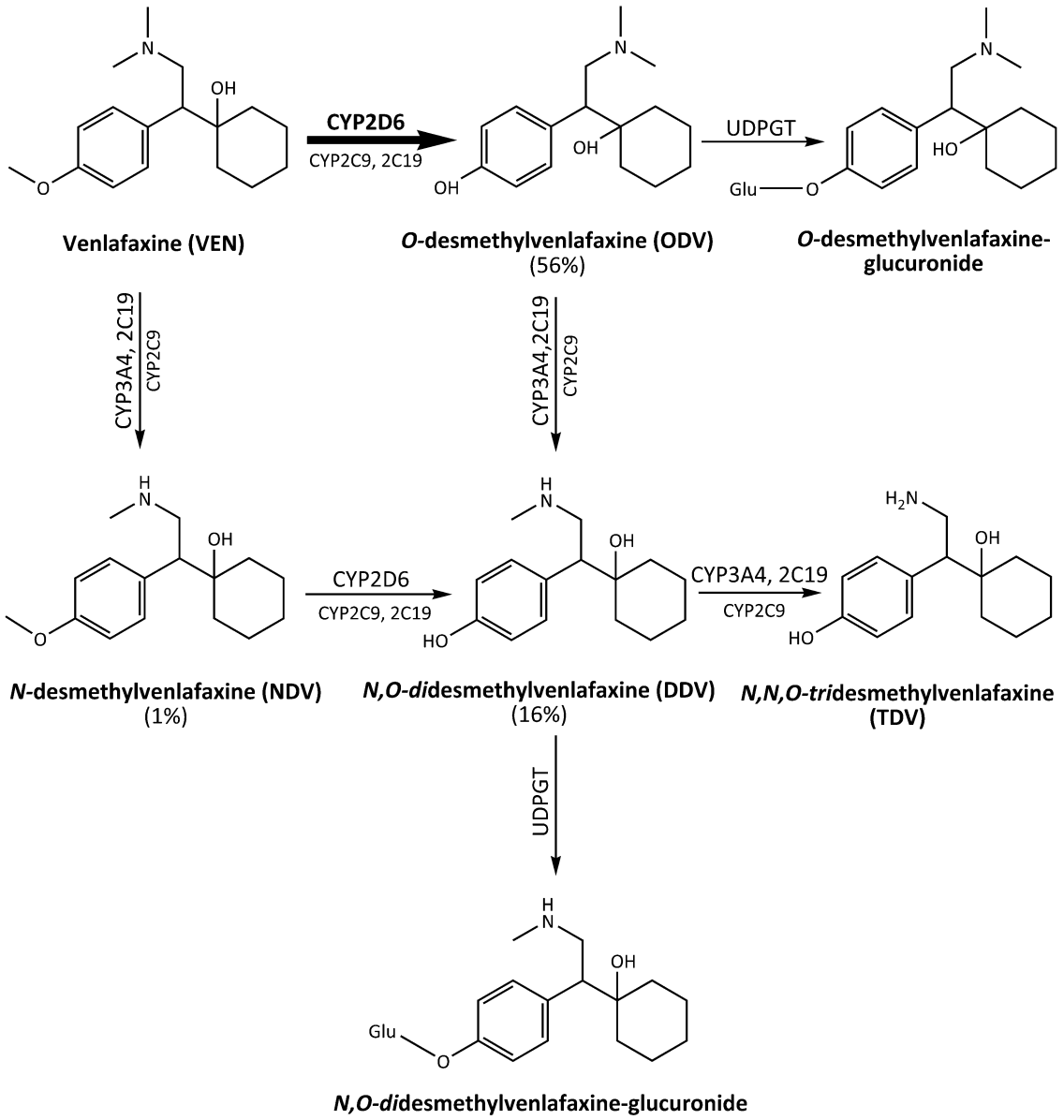


Figure I.2.5 Metabolic pathway of venlafaxine (VEN) in humans (68,127,132,135–137). The bold arrow indicates the major metabolic pathway. The main metabolizing enzyme(s) of each pathway is/are indicated above the arrows, at superior size. CYP, cytochrome P450 and UDPGT, UDP-glucuronosyltransferase.

Of note, VEN is available in two type of formulations (IR and XR), which differ in the release profiles of VEN and, consequently, in its pharmacokinetics (130). VEN XR is usually administered once-daily and the drug has a prolonged absorption profile, resulting in a lower maximum plasma concentration (C_{max}) when compared with IR (Table I.2.4); even so the total absorption of VEN appears to be equivalent independently of the formulation used (127,130). The XR formulation is considerably better than the IR

formulation in terms of patient convenience and compliance and fluctuation index of plasma concentrations. Actually, the lower peak-to-trough fluctuation in plasma VEN concentrations has been shown to improve the drug's tolerability profile and reduces the C_{max} related side effects (e.g. nausea and dizziness) (127,130,138–140).

Once VEN and ODV have entered into the systemic circulation, they are widely distributed throughout the body. Bearing in mind the estimated values for the steady-state apparent volume of distribution of VEN and ODV (Table I.2.4), it is evident that both compounds are well distributed beyond the total body water (119,141). The limited extent of binding of VEN and ODV to human plasma proteins (Table I.2.4, 27 % and 30 %, respectively) can also contribute for the large apparent volumes of distribution exhibited by both compounds (119,141); hence, the occurrence of drug interactions in plasma protein binding involving VEN is unlikely (127). Also under this context, it is worthy of note that VEN and ODV cross the placenta and they are also extensively distributed into breast milk (141–143); in truth, the area under the concentration-time curve (AUC) was shown to be approximately 3- to 5-fold higher in breast milk than in maternal plasma (141).

In turn, various authors have claimed that VEN and its main metabolite (ODV) are P-gp substrates (87,144–148). This information is especially important seeing that P-gp is expressed in the intestinal epithelium and brain vascular endothelium, which can influence the oral bioavailability and the distribution of VEN and ODV to the brain (biophase) (144,146,147). Indeed, Karlsson *et al.* found that the brain concentrations of VEN and some of its metabolites were 2- to 4-fold times higher in P-gp knockout versus wild-type mice. These data show that the expression of P-gp plays an important role in limiting brain access of VEN and its metabolites (149). Therefore, it is likely that differences in expression and function of the P-gp are able to explain, at least in part, the inter-individual variability observed in clinical outcomes of patients receiving VEN, such as adverse effects, therapeutic failure and even discrepancies between plasma levels and clinical response (144,146,147). Additionally, studies performed in Caco-2 cells (150) and in human brain endothelial cells (blood-brain barrier model) (144) clearly demonstrated that VEN is, on the contrary to ODV, an inducer of the expression of drug efflux transporter proteins, among them P-gp.

Regarding metabolism, VEN undergoes extensively hepatic biotransformation catalysed by CYP isoenzymes, and *in vitro* and *in vivo* studies have indicated that CYP1A2,

CYP2D6, CYP2C9, CYP2C19 and CYP3A4 are the CYP isoforms involved. Specifically, the *O*-demethylation of VEN to ODV is the main metabolic pathway in humans, being approximately 56 % of dose metabolized through this process, which is primarily mediated by CYP2D6 (95,127,131,133,151). Consequently, the plasma concentrations of the active metabolite ODV are usually higher (2- to 3-fold) than those of VEN in man. Other minor metabolic routes implicated in the oxidative metabolism of VEN and its metabolites are additional *N*- and *O*-demethylation reactions (127,130,132,133). *N*-demethylation reactions appear to be, at least partially, mediated via CYP3A4 (95,127,131,133,152). Several studies have shown that CYP2C9 and CYP2C19 also participate in the *O*- and *N*-demethylation metabolic pathways of VEN (117,118,127,130,135,137,152–154). Furthermore, there is evidence that the metabolites ODV and DDV participate in conjugation (phase II) metabolic reactions, leading to the formation of the corresponding aryl *O*-glucuronide metabolites (136).

VEN and its related compounds are primary excreted by kidneys (92.1 %). More specifically, Howell and collaborators demonstrated that VEN is excreted in human urine as unchanged VEN (4.7 %), unconjugated ODV (29.4 %), conjugated ODV (26.4 %), unconjugated DDV (9.8 %), conjugated DDV (6.2 %), NDV (1.0 %) and TDV (1.0 %). Overall, the terminal elimination half-life of VEN is approximately 5 hours and that for ODV is about 11 hours (31,35). Particularly, under oral multiple-dose therapy, the steady-state plasma concentrations of VEN are reached within 3 days. Pharmacokinetics of VEN and its active metabolite (ODV) is linear in the dose range of 75-450 mg/day (Table I.2.4) (129,130).

Finally, as abovementioned, VEN is a well-known substrate of CYP isoenzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) and P-gp efflux transporter, as well as a weak inhibitor of CYP2D6 and inducer of P-gp. Therefore, such as FLU and PAR, VEN may also be involved in clinically relevant pharmacokinetic-based drug interactions with other co-administered drugs affecting these proteins, both as precipitant and as object drug (95,127,151,130,131,133,144–148). On this matter, Magalhães *et. al* conducted a detailed and comprehensive review of the pharmacokinetic- and pharmacodynamic-based drug interactions involving VEN, particularly addressing their clinical relevance (65). They concluded that VEN is one of the safer antidepressants in terms of the propensity to be involved in clinically significant drug-drug interactions, being, thereby, a good alternative

to SSRIs in polymedicated patients, chiefly if they are taking narrow therapeutic index drugs. In fact, VEN presents a lower potential to precipitate drug-drug interactions compared to other antidepressant drugs, namely FLU and PAR, given the weak effects on the CYP isoenzymes (Table I.2.4) (65).

I.2.2.2.1.2 PHARMACODYNAMICS

VEN is clinically used as a racemic mixture of two pharmacologically active enantiomers [*S*-(+)-VEN and *R*-(-)-VEN], which present similar absorption and disposition properties (126). The *S*-(+)-enantiomer primarily acts as a serotonin reuptake inhibitor, whereas the *R*-(-)-enantiomer inhibits both serotonin and norepinephrine reuptake (131,151). Because of that, VEN is more potent as serotonin reuptake inhibitor rather than as norepinephrine reuptake inhibitor, and essentially acts as a serotonin reuptake inhibitor under low daily doses (75 mg/day), becoming a dual-acting antidepressant only under daily doses above 150 mg/day (95,102,131,155,156). Additionally, VEN is also a weak inhibitor of dopamine reuptake, but it does not inhibit monoamine oxidase, neither has significant affinity for α_1-adrenergic, muscarinic cholinergic, H_1 histaminergic, benzodiazepine or opioid receptors (95,130).

As a result, VEN has a low potential to cause anticholinergic and orthostatic hypotensive adverse effects, as well as sedation or weight gain. In fact, VEN has been proposed to have a favourable tolerability profile comparatively to other antidepressant drugs, specifically TCAs and tetracyclic antidepressants (151). However, this drug may suppress the rapid eye movement sleep and increase the wake time, but particularly significant is the fact that VEN may elevate the blood pressure (101,130,157). In addition, VEN impairs sexual function and may increase the risk of upper gastrointestinal bleeding, although the absolute risk of bleeding appears to be small. Overdoses of VEN can lead to hypertension, cardiac arrhythmias, seizures, serotonin syndrome and death (101). Recently, VEN was found among the most efficacious antidepressant drugs but, on the other hand, between the less tolerable ones (92).

To end, such as the SSRIs and other SNRIs, VEN may also pharmacodynamically interact with serotoninergic drugs, which may result in serotonin syndrome, as well as with drugs that interfere with haemostasis, augmenting the anticoagulant or

antiaggregant effects (65,158). Detailed description and discussion of these clinically relevant pharmacodynamic-based drug interactions is provided elsewhere (65).

I.3 HIGH INTER-INDIVIDUAL VARIABILITY AND POOR CLINICAL OUTCOMES WITH ANTIDEPRESSANT DRUGS

I.1.3 HIGH INTER-INDIVIDUAL VARIABILITY AND POOR CLINICAL OUTCOMES WITH ANTIDEPRESSANT DRUGS

I.1.3.1 GENERAL OVERVIEW

Unfortunately, despite the large armamentarium of older and newer antidepressants currently available, a significant proportion of patients with depressive disorders remains inadequately treated, especially due to the high inter-individual variability and poor clinical outcomes associated with antidepressant drugs (147,159,160). Only about 1/3 of patients reach complete symptom remission after the first antidepressant trial, while 1/3 of patients do not respond to antidepressant drug treatments (drug-resistance depression) (161). In turn, the delayed onset of antidepressant therapeutic response may increase the risk of non-compliance and even of suicide. Furthermore, the relapse rate of depression and the frequency of antidepressant drugs-related adverse effects have been found to be very high. More than 40 % of patients experience relapse episodes and around 40-90 % experience adverse effects, with up to 43 % of patients discontinuing the antidepressant treatment for this reason (71,162).

Actually, depressive patients treated with antidepressant drugs, including with FLU, PAR and VEN, have exhibited large inter-individual variability in drug pharmacokinetic and pharmacodynamic outcomes (efficacy *versus* adverse effects). This inter-individual variability has been extensively studied in a pharmacogenetic perspective, focused on individual genetic factors related to drug pharmacokinetics and pharmacodynamics. However, pharmacogenetics alone has not been able to fully explain such clinical outcomes. The co-effects of individual non-genetic factors, such as co-medication and co-morbidities, have been pointed out as the reason for that (69,87,167–170,95,123,147,160,163–166). The next sections will provide a comprehensive review of what is known about the influence of genetic and non-genetic factors on the pharmacokinetics and pharmacodynamics of antidepressant drugs, focusing on FLU, PAR and VEN.

I.1.3.2 GENETIC FACTORS

Evidence from pharmacogenetics research suggests that genetic factors may contribute for about 50 % of the clinical outcomes of antidepressant drugs (160,171). In fact, pharmacogenetics is currently one of the most promising approaches in clinical psychiatric. The pharmacogenetics aims at identifying genetic factors that determine relevant variability in the clinical outcomes of antidepressant drugs or, in other words, to identify genetic biomarkers. Several works have revised and updated the pharmacogenetics of antidepressant drugs over the years (69,87,147,161,162). Overall, pharmacogenetics has explored genetic factors related to the pharmacokinetics and pharmacodynamics of antidepressant drugs. Pharmacodynamic-related genes are those whose products are known to be involved in the mechanisms of action and are more directly involved in antidepressant efficacy. On the other hand, drug efficacy is also influenced by complex pharmacokinetic processes that regulate the absorption, distribution and elimination of a drug and, consequently, its concentration in the site of action. Genes involved in drug pharmacokinetics can be classified in those coding for proteins involved in drug metabolism and genes coding for proteins that guarantee drug transport through the body (e.g. in absorption, transport through the blood stream and transport through the blood-brain-barrier). Among them, the genetic polymorphisms of the CYP2C9, CYP2C19, CYP2D6 and P-gp have deserved particular attention in the last years.

I.1.3.2.1 P-GLYCOPROTEIN

P-gp is a member of the ATP-binding cassette superfamily of membrane transport proteins encoded by the *ABCB*1 gene, also known as the multidrug resistance protein 1 (*MDR1*) gene. Like other ABC-transporters, P-gp acts as an energy-dependent efflux pump that uses ATP hydrolysis as an energy source for the active transport of its substrates across cell membranes (69,87,160–162). P-gp is expressed in the luminal membrane of brain capillary endothelial cells at the blood-brain barrier level, but also in other tissues/organs needing special protection (e.g. placenta and testis) or being responsible for the elimination of xenobiotics/toxins (e.g. liver, kidney and intestine). At the blood-

brain barrier, P-gp can be thought as a safety guard that protects the brain against xenobiotics/toxins (including drugs) by keeping low brain concentrations of its substrates. Due to its function as an efflux transporter in the blood–brain barrier, P-gp is a candidate biomarker for response to central nervous system-active drugs, such as antidepressants (87). In fact, P-gp could be a limiting factor for the bio-access of substrate antidepressant drugs into the brain, the target site of antidepressant action (69,87,160–162). *In vitro* and *in vivo* preclinical studies have revealed that the majority of antidepressant drugs are P-gp substrates and, therefore, brain concentrations of these drugs may depend on P-gp functional activity. As previously discussed, PAR and VEN have been classified as P-gp substrates, while the P-gp status of FLU is not clear (87,144–148). At this level, it has been hypothesized that a high P-gp expression at blood-brain barrier and/or a higher functional activity may lead to lower and often insufficient brain concentrations of P-gp substrate antidepressant drugs. On the other hand, lower P-gp expression or reduced activity may facilitate the access of P-gp substrate antidepressant drugs into the brain (68,87).

Recently, an extensive review provided the most updated clinical evidence available regarding these matters. This work summarized and discussed the results of 32 clinical studies that investigated the impact of *ABCB1* polymorphisms on the clinical efficacy and/or tolerability of antidepressant drugs and its potential as biomarkers. At this level, the most well-studied genetic variants are the exonic SNPs: *rs1128503 (C1236T)*, *rs2032582 (G2677T)* and *rs1045642 (C3435T)* (69,87,161). Accordingly, these three *ABCB1* genetic polymorphisms have been investigated in clusters within the same study as haplotype analysis. Nevertheless, the effects of these polymorphisms on the functional activity of P-gp are not completely clear and there are a lot of controversial findings. Table I.3.1 resumes the most common genetic polymorphisms of the *ABCB1* gene and corresponding frequency in Caucasians.

Table I.3.1 Summary of the most common genetic polymorphisms of the *ABCB1* gene and corresponding frequency in Caucasians (172).

Gene	SNP(s)	rs code(s)	Functional activity	Allele frequency in Caucasians [% (95% CI)]	
ABCB1	*1236C>T*	rs1128503	Not clear	*C*	58.4 (55.4-61.5)
				T	41.6 (38.5-44.6)
	2677G>T/A	rs2032582	Not clear	*G*	57.3 (54.2-60.3)
				T	41.0 (37.9-44.0)
				A	1.8 (1.0-2.6)
	3435C>T	rs1045642	Not clear	*C*	51.8 (48.7-54.9)
				T	48.2 (45.1-51.3)

ABCB1, P-glycoprotein gene; SNP, single nucleotide polymorphism.

Specifically, the *ABCB1 rs1045642* SNP (*C3435T*) has been associated with human P-gp expression in the intestine, with *TT* carriers having more than a two-fold lower P-gp expression in the duodenum than *CC* carriers. At this level, VEN and escitalopram were found to display better remission rates among carriers of the T-allele. However, other studies did not find an effect of this polymorphism on the response to P-gp substrate drugs, such as PAR and citalopram (87). Additionally, carriers of the *ABCB1* r*s1045642* (*C3435T*) *TT* genotype were found to achieve remission with a significantly lower dose of escitalopram than *CC* or *CT* carriers. In turn, the *T* allele of *ABCB1 rs1045642 (C3435T)* SNP was associated with a greater likelihood of switching the first prescribed antidepressant drug and with a higher frequency of occurrence of postural hypotension in nortriptyline-treated patients (87). Contrarily, other studies were unable to replicate these achievements or reported an opposite effect (87). Similarly, the *T* allele of the *ABCB1 rs2032582 (G2677T)* SNP has also been associated with an improved clinical efficacy or worse tolerability profile, but with controversial findings as well. For instance, the *ABCB1 rs2032582 (G2677T)* SNP was shown to predict the antidepressant response of PAR and FLU in children and adolescents, as well as of VEN and FLU in adults. Nonetheless, no effect of this genetic polymorphism on clinical efficacy of antidepressant drugs has been found in several other clinical studies. Regarding the *ABCB1 rs1128503 (C1236T)* SNP*, TT*

genotype was associated with a significantly lower dose of escitalopram needed for remission and with SSRI-induced sexual dysfunctions in females; *T* allele has also been correlated to premature antidepressant discontinuation.

Importantly, Brückl *et al.* concluded that genetic variants within the *ABCB1* gene may be simultaneously biomarkers of drug clinical efficacy and tolerability (87). Based on the evidence available so far, they classified the *ABCB1 rs1128503 (C1236T), rs2032582 (G2677T)* and *rs1045642 (C3435T) T* allele as an allele responsible to improve the antidepressant therapeutic response, but also as an allele responsible to decrease the tolerability. On the other hand, the *C* allele was considered as a poor response allele. Indeed, *T* allele has been associated with lower P-gp expression at the blood-brain barrier and easier access of P-gp substrate antidepressant drugs to the brain. Thus, higher antidepressant brain levels may not necessarily lead to better clinical outcomes. At a certain point, they could compromise drug tolerability (87). These considerations help to explain why the *ABCB1 1236TT-2677TT-3435TT* haplotype has been associated with poor psychiatric outcomes, specifically violent suicide attempts (173).

As abovementioned, these three genetic polymorphisms are the most studied genetic factors related to the *ABCB1* gene (86). However, the clinical evidence supporting associations between these genetic polymorphisms and the clinical outcomes of antidepressant drugs is still modest. Several experts in the field of pharmacogenetics of antidepressant drugs have claimed for further studies, especially in the presence of other genetic and non-genetic modulators of the clinical outcomes (69,87,161).

I.1.3.2.2 CYTOCHROME P450

The CYP superfamily is a class of enzymes with a major role in oxidation and reduction metabolic reactions of both endogenous and xenobiotic substances (69,162,174). These CYP isoenzymes are responsible for phase I oxidative metabolism of approximately 80 % of the commonly used drugs (162). More than 50 CYP isoenzymes are known so far. However, CYP2D6, CYP2C19 and CYP2C9 are the main isoenzymes involved in the metabolism, drug-drug interactions and inter-individual variability issues of the antidepressant drugs, including FLU, PAR and VEN (161,174).

In fact, genes encoding CYP isoenzymes are highly polymorphic, with genetic polymorphisms occurring in 1–30 % of people, largely dependent on ethnicity. At the level of *CYP2C9, CYP2C19* and *CYP2D6* genes, a large number of genetic polymorphisms are currently known to influence the enzyme functional activity, leading to different metabolizer groups (phenotypes) with increased, decreased or absent drug metabolic capacity. These have been, in turn, associated with clinically relevant inter-individual differences in the antidepressant outcomes, namely with FLU, PAR and VEN (69,161,162,174–177). The next sections will provide a comprehensive overview of the pharmacogenetics of important CYP isoenzymes involved in the metabolism of FLU, PAR and VEN, focusing particularly on the CYP2C9, CYP2C19 and CYP2D6.

I.1.3.2.2.1 CYP2C9

CYP2C9 is one of the most abundant CYP isoenzymes in the human liver, representing around 20 % of the total hepatic CYP content. This isoenzyme is involved in the metabolism of about 15 % of all drugs. The *CYP2C9* gene is located on the chromosome 10q24 in a multigene cluster containing the other CYP2C subfamily members (CYP2C8, CYP2C18 and CYP2C19) (178). Like for the other main isoenzymes involved in the metabolism of antidepressant drugs (CYP2C19 and CYP2D6), this CYP isoenzyme is highly polymorphic. More than 50 *CYP2C9* allelic variants are described so far (February, 2019) (179). The two most common genetic variants associated with reduced enzyme activity are *CYP2C9*2* and *CYP2C9*3*, whereas *CYP2C9*1* is the wild-type allele (174,178). For most substrates, *CYP2C9*3* heterozygous individuals have approximately 50 % of the wild-type total oral clearance and *CYP2C9*3* homozygous individuals have a 5- to 10-fold reduction (174). Overall, genetic polymorphisms in the *CYP2C9* gene result in a reduced enzyme activity in 0.7 % of Caucasians (174).

I.1.3.2.2.2 CYP2C19

CYP2C19 is responsible for the metabolism of approximately 10 % of the commonly used drugs. *CYP2C19* gene is located on the chromosome 10q24.1–q24.3 and it is also highly polymorphic. Over 35 allelic variants and subvariants have been identified

until now (February, 2019) (179). However, the most frequent allelic variants are *CYP2C19*1*, *CYP2C19**2 and *CYP2C19**17. *CYP2C19*1* encodes a normal function enzyme, while *CYP2C19*2* is the most common no functional allele followed by *CYP2C19*3*. In turn, the *CYP2C19*17* allele is defined by a variant in the promoter region, resulting in enhanced gene transcription and in an increased metabolic capacity (178,180). Noteworthy, significant differences in the *CYP2C19* allele frequencies have been observed among populations (180).

I.1.3.2.2.3 CYP2D6

Although CYP2D6 accounts for only about 5 % of the total hepatic CYP content, this isoenzyme plays a major role in drug metabolism, being partially or entirely responsible for the oxidative biotransformation of up to 25 % of the commonly prescribed drugs (177,178). The *CYP2D6* gene is located on the chromosome 22q13.1 and it is highly polymorphic, with over 100 known allelic variants and subvariants identified (February, 2019) (179). *CYP2D6* alleles have been extensively studied across populations and significant differences in allele frequencies have been reported (177,178,180). The most commonly reported alleles are categorized into functional groups as follows: normal function (e.g. *CYP2D6*1* and **2*), decreased function (e.g. *CYP2D6*10* and **41*), and no function (e.g. *CYP2D6*3 to*6*). *CYP2D6* gene is also subject to deletions, duplications, or multiplications. For example, *CYP2D6*5* represents a gene deletion, whereas gene duplications and multiplications are denoted by "xN" (e.g. *CYP2D6*1*xN with xN representing the number of copies). In Europe, 95–99 % of the genotype-predicted poor metabolizers (gPMs) are detected by screening the main null alleles *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*6* and the gene deletion *CYP2D6*5*. The common deficient alleles are *CYP2D6*9*, *CYP2D6*10*, *CYP2D6*17* and *CYP2D6*41* (177,178,180).

I.1.3.2.2.4 CYTOCHROME P450 PHARMACOGENETIC TESTS: GENOTYPING AND PHENOTYPING

Pharmacogenetics not only intends to explore genotype or genotype-predicted phenotype (gPH)-drug outcomes associations, but also phenotype-drug outcomes

associations. Apart from the classic genotyping assays, phenotyping assays are also available for the CYP isoenzymes and even for some drug transporters like P-gp. These may be used in pharmacogenetic studies with or without genotyping tests.

Regarding CYP genotyping, clinical laboratories usually test for the more frequently observed genetic variants of the gene and translate the results into star-allele (*) nomenclature. Each star-allele, or haplotype, is defined by a specific combination of SNPs and/or other genetic variants within the specific gene locus. Genotyping results are reported as the summary of inherited maternal and paternal star-alleles referred to as a diplotype (e.g. *CYP2D6*1/**2 and *CYP2C19*1/*1*). However, different clinical laboratories may use varying methods to predict phenotype from genotype data (180). Overall, the individuals can be phenotypically classified into four major metabolizer phenotypes predicted from genotype (gPH) for each isoenzyme: gPMs, those who lack functional isoenzyme; genotype-predicted intermediate metabolizers (gIMs), those who are heterozygous for a defective allele or carry two alleles that cause reduced activity; genotype-predicted extensive metabolizers (gEMs), those who have two normal alleles; and genotype-predicted ultra-rapid metabolizers (gUMs), those who carry more than two functional gene copies or functional polymorphisms associated with increased gene expression or protein functionality. The gEM phenotype is usually the most frequent and corresponds to normal metabolic function, whereas the other gPHs display a reduced (gPMs and gIMs) or increased (gUMs) metabolic activity (152,153,176,181). At this point, it is worth mentioning that one of the strategies that has been used to translate and predict the CYP phenotype from genotype determined by genotyping is the Activity Score (AS) system. Briefly, the AS system is a numerical scoring system where each allele is scored according to its known functional activity (null, decreased, normal or increased). The total AS for the diplotype is obtained by the sum of the score for both alleles, which is then used to get the corresponding gPH (182,183). Table I.3.2 summarizes the most common genetic polymorphisms and alleles for the *CYP2C9*, *CYP2C19* and *CYP2D6* genes and corresponding functional activity level, AS properties and frequency in Caucasians.

Table I.3.2 Summary of the most common genetic polymorphisms of the *CYP2C9*, *CYP2C19* and *CYP2D6* genes and corresponding functional activity, Activity Score (AS) properties and frequency in Caucasians (172,184).

Gene	Allelic variant	SNP(s)	rs code(s)	Functional activity	AS	Allele frequency in Caucasians [% (95% CI)] [a]
CYP2C9	**1^{wt}*			Normal	1	80.3 (77.9-82.8)
	**2*	*430C>T*	rs1799853	Decreased	0.5	12.4 (10.3-14.4)
	**3*	*1075A>C*	rs1057910	Null	0	7.3 (5.56-8.8)
	**6*	*818delA*	rs9332131	Null	0	0.0
CYP2C19	**1^{wt}*			Normal	1	63.0 (60.0-66.0)
	**2*	*681G>A*	rs4244285	Null	0	14.5 (12.3-16.7)
	**3*	*636G>A*	rs4986893	Null	0	0.0
	**4*	*1A>G*	rs28399504	Null	0	0.1 (0.1-0.3)
	**5*	*1297C>T*	rs56337013	Null	0	0.0
	**17*	*806C>T*	rs12248560	Rapid	2[a]	22.4 (19.8-25.0)
CYP2D6	**1^{wt}*			Normal	1	40.1 (37.7-42.5)
	**2*	*1584C>G, 2850C>T*	rs1080385, rs16947	Normal	1	17.0 (15.2-18.9)
	**3*	*2549del>A*	rs35742686	Null	0	1.4 (0.9-2.1)
	**4*	*1846G>A 100 C>T*	rs3892097, rs1065852	Null	0	19.0 (17.2-21.0)
	**5*	*whole-gene deletion*		Null	0	1.6 (1.1-2.4)
	**6*	*1707delT*	rs5030655	Null	0	0.9 (0.5-1.5)
	**10*	*100C>T*	rs1065852	Decreased	0.5	2.2 (1.6-3.0)
	**17*	*1023C>T 2850C>T*	rs28371706, rs16947	Decreased	0.5	0.9 (0.6-1.5)
	**29*	*3183G>A*	rs59421388	Decreased	0.5	0
	**35*	*1584 C>G 31G>A*	rs1080385, rs769258	Normal	1	5.3 (4.3-6.5)
	**41*	*2988G>A*	rs28371725	Decreased	0.5	7.5 (6.3-9.0)
	**1xN*			Rapid	2[a]	0.6 (0.3-1.1)
	**2xN*			Rapid	2[a]	2.9 (2.2-3.9)
	**35xN*			Rapid	2[a]	

Activity Score (AS) system

AS	Genotype-predicted phenotype	Combined CYP2C9-2C19-2D6 AS (CAS)[b]	Combined metabolic capacity
≤ 0.5	Poor metabolizer (gPM)	< 2	Decreased
1.0 or 1.5	Intermediate metabolizer (gIM)	2	Normal
2.0	Extensive metabolizer (gEM)	> 2	Increased
> 2.0	Ultra-rapid metabolizer (gUM)		

CYP, cytochrome P450; SNP, single nucleotide polymorphism; ^{wt}, wild-type allele.
[a] in positive cases for the CYP2D6 multiplication the AS corresponds to the N value, i.e. the number of times that the CYP2D6 allele is multiplied; [b] calculated as: CYP2C9 AS + CYP2C19 AS + CYP2D6/3

Naturally, this phenotypic classification based on the genotype does not exactly reflect the real functional activity status (phenotype). This because it does not consider that some alleles are only partially active, that there are different degrees of activity between alleles of the same phenotype group and that some alleles show different activity, depending on the drug metabolized. Then, this system is only based on genetic factors, but non-genetic factors also contribute to determine the real metabolic phenotype (see section I.1.3.3) (174). Thus, differences between the gPH and the real phenotype may exist (153). In accordance with Crisafulli *et al.,* the genotype of polymorphic CYP isoenzymes is frequently discrepant of the real metabolic phenotype and, therefore, the genotyping and phenotyping complement to each other (160,185–187). Phenotyping can be performed to determine the level of functional activity of a specific isoenzyme (or even of a transporter), through the use of selective metabolic probes, or ideally using the drug itself (188–191). Currently, the accepted phenotyping procedure consists on the determination of metabolic ratios (proportion between the amounts of unchanged drug and metabolite mediated by a specific isoenzyme) in body fluids, usually plasma and urine, within a certain time following a single-dose administration of a probe drug. These procedures require the availability of appropriate analytical methodologies able to quantify both compounds, probe drug and metabolite (153,174,192–194). Various pharmacologic agents selectively metabolized by CYP2D6 have been used to assess the CYP2D6 metabolic phenotype (e.g. debrisoquine, sparteine, tramadol, bufuralol, metoprolol, and dextromethorphan) (152,193,195). Moreover, recent studies have demonstrated that the plasma or serum ODV/VEN ratio is a useful marker of the CYP2D6 metabolic capacity (151,153,193,196,197). Regarding the other isoenzymes, there are equally probe compounds that have been used for phenotyping procedures, such as mephenytoin and omeprazole for CYP2C19 (153) and losartan for CYP2C9 (198). These phenotyping approaches have also been used in pharmacogenetics research to investigate phenotype-drug outcomes associations and putative phenotypic biomarkers. For the sake of clarity, gPHs determined by genotyping will be hereafter signalized with the prefix “g” (e.g. gPMs) and phenotypes determined by phenotyping will be presented with no prefix (e.g. PM), as proposed by Shah *et al.* (176).

I.1.3.2.2.5 CYTOCHROME P450 PHARMACOGENETICS: FLUOXETINE, PAROXETINE AND VENLAFAXINE

The pharmacogenetics of antidepressant drugs regarding CYP isoenzymes has been constantly revised and, consequently, specific guidelines for therapeutic interventions based on the genotypes have been proposed, namely for FLU, PAR and VEN (69,147,161,162,174,177,178,180,199). Numerous studies have investigated the influence of genetic factors related to CYP2D6 on the pharmacokinetics (drug concentrations) of FLU, PAR and VEN. Despite this, controversial findings exist at this level and the influence on the clinical outcomes (efficacy and safety) has been underexplored (69,160,161,200). On the other hand, few studies have investigated the influence of pharmacogenetic factors related to CYP2C9 and CYP2C19 on the treatment with FLU, PAR and VEN (either on the pharmacokinetics, as on the clinical outcomes). Table I.3.3 summarizes the pharmacogenetics of FLU, PAR and VEN at the level of CYP2D6, CYP2C9 and CYP2C19, as well as the related therapeutic recommendations that have been proposed. The next sections will address these issues in detail for FLU, PAR and VEN.

Table I.3.3 Summary of the pharmacogenetics of fluoxetine, paroxetine and venlafaxine at the level of CYP2D6, CYP2C9 and CYP2C19 and related therapeutic recommendations.

Fluoxetine				
Phenotype [a]	**Pharmacokinetics impact**	**Pharmacodynamics impact**	**Therapeutic recommendation**	**Refs**
CYP2D6 PM	↑ [FLU] →EMs	Insufficient data. Not clear	None. Insufficient evidence. Monitor CYP2D6 UMs and PMs treated with FLU or select an alternative SSRI not extensively metabolized by CYP2D6	(180,201, 202)
	↑ [*S*-FLU] and ↓ [*S*-NFLU] → EMs Impact on active portion (FLU+NFLU) not clear			
CYP2D6 IM	Insufficient data. Not clear			(180)
CYP2D6 UM	Insufficient data. Not clear			(180)
CY2C9 IM	↑ [FLU] and [FLU+NFLU] → EMs			(203)
CYP2C9 non-wild-type	↑ [*R*-FLU] and [*S*-FLU + *R*-FLU + *S*-NFLU] →EMs			(97)

Table I.3.3 Summary of the pharmacogenetics of fluoxetine, paroxetine and venlafaxine at the level of CYP2D6, CYP2C9 and CYP2C19 and related therapeutic recommendations.

Fluoxetine

Phenotype [a]	Pharmacokinetics impact	Pharmacodynamics impact	Therapeutic recommendation	Refs
Remaining CYP2C9 and CYP2C19 phenotypes	Insufficient data. Not clear			(69,161,174)

Paroxetine

Phenotype [a]	Pharmacokinetics impact	Pharmacodynamics impact	Therapeutic recommendation	Refs
CYP2D6 PM	↑ [PAR] → EMs	↑ risk of adverse effects → other metabolic groups	Select an alternative SSRI not extensively metabolized by CYP2D6 or reduce 50 % the recommended starting dose and titrate to response	(201,204–206)
CYP2D6 IM	Insufficient data. Not clear		None. Insufficient evidence	(180)
CYP2D6 UM	↓ [PAR] → CYP2D6 EMs	Insufficient data; it may be a risk factor for poor therapeutic response or therapy failure	Select an alternative SSRI not extensively metabolized by CYP2D6	(201,207–209)
CYP2C9 and CYP2C19	Insufficient data. Not clear		None. Insufficient evidence	(69,161,174)

Venlafaxine

Phenotype [a]	Pharmacokinetics impact	Pharmacodynamics impact	Therapeutic recommendation	Refs
CYP2D6 PM	↑ [VEN] and [NDV] and ↓ [ODV] → EMs	↑ risk of adverse effects and poor therapeutic response	Select an alternative drug or adjusting dose to clinical response and monitoring [VEN] and [ODV]	(117,134,153,154,193,210–213)

Table I.3.3 Summary of the pharmacogenetics of fluoxetine, paroxetine and venlafaxine at the level of CYP2D6, CYP2C9 and CYP2C19 and related therapeutic recommendations.

Venlafaxine

Phenotype [a]	Pharmacokinetics impact	Pharmacodynamics impact	Therapeutic recommendation	Refs
CYP2D6 IM	↑ [VEN] and [NDV] → EMs	Insufficient data. Not clear		(68,165)
CYP2D6 UM	↑ [NDV] → EMs	Poor therapeutic response	Titrate the dose to a maximum of 150 % of the normal dose or select an alternative drug	(159,214) (177,215)
CYP2C9 and CYP2C19	Insufficient data. Not clear		None. Insufficient evidence	(68)

CYP, cytochrome P40; [FLU], fluoxetine concentrations; [FLU+NFLU], fluoxetine + norfluoxetine concentrations; [*S*-FLU], *S*-fluoxetine concentrations; [*R*-FLU], *R*-fluoxetine concentrations; [*S*-NFLU], *S*-norfluoxetine concentrations; [*S*-FLU + *R*-FLU + *S*-NFLU], *S*-fluoxetine + *R*-fluoxetine + *S*-norfluoxetine concentrations, [PAR], paroxetine concentrations; [VEN], venlafaxine concentrations; [ODV], *O*-desmethylvenlafaxine concentrations; [NDV], *N*-desmethylvenlafaxine; ↑, higher; ↓, lower; →, compared to; PM, poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer; UM, ultra-rapid metabolizer.

[a] genotype-predicted phenotype or phenotype determined by phenotyping.

I.1.3.2.2.5.1 FLUOXETINE

CYP2D6 gPMs have been demonstrated to possess significantly higher FLU plasma concentrations than gEMs. However, few data are available describing how CYP2D6 phenotype status influences the concentrations of the active portion (FLU + NFLU) over the time, or if an imbalance between FLU and NFLU concentrations caused by CYP2D6 phenotype status affects the clinical outcomes (efficacy and/or safety) (180). The same is applicable to the influence of genetic factors related to CYP2C9 and CYP2C19. Thus, no specific gene-based dosing recommendations are available for FLU. Nevertheless, it has been recommended to monitor CYP2D6 gUMs and gPMs patients treated with FLU or to select an alternative SSRI not extensively metabolized by CYP2D6 (Table I.3.3) (180).

In detail, Llerena *et al.* found that steady-state FLU concentrations and FLU/NFLU ratios were negatively correlated with the number of CYP2D6 active genes in Caucasian psychiatric patients. Furthermore, among the CYP2D6 gEMs patients (two active alleles),

plasma concentrations of FLU and active moiety (FLU+NFLU) were significantly higher in CYP2C9 gIMs patients (*CYP2C9*1/*2* and *CYP2C9*1*) compared to CYP2C9 gEMs patients (*CYP2C9*1/*1*) (203). Genetic status of CYP2D6 was also correlated to FLU and PAR concentrations in Caucasian patients, where lower steady-state plasma concentrations of FLU were observed in CYP2D6 gEMs compared to gPMs (201). On the other hand, other authors have reported an effect of the CYP2D6 genotype only on the metabolism of the *S*-FLU enantiomer (97,174,202). Steady-state concentrations of *S*-FLU and *S*-NFLU were found to be higher and lower, respectively, in CYP2D6 gPMs when compared with gEMs, indicating that the CYP2D6 is involved in the demethylation of FLU to NFLU, with stereoselectivity toward the *S*-enantiomer (202). Scordo *et al.* evaluated the influence of CYP2D6, CYP2C9 and CYP2C19 polymorphisms on the steady-state plasma concentrations of FLU and NFLU enantiomers; they found a very low plasma concentration of *S*-NFLU in the only one CYP2D6 gPM included in the study and higher *S*-NFLU/*S*-FLU ratios in gEMs compared to gIMs. Moreover, they also observed that among the CYP2D6 gEMs patients, those who were CYP2C9 gEMs had lower *R*-FLU concentrations and lower *S*-FLU + *R*-FLU + *S*-NFLU levels (main active portion) compared to CYP2C9 non-wild-type patients (97). Lastly, Gassó *et al.* investigated the effect of the *CYP2D6*, *CYP2C9* and *ABCB1* genotypes on the steady-state plasma concentrations of FLU and *S*-NFLU and clinical improvement in children and adolescent patients. In agreement with the previous findings, they observed a negative correlation between FLU/*S*-NFLU ratio and the number of active CYP2D6 alleles (98). Nonetheless, no influence of the *CYP2C9* genotype on the FLU concentrations was found by this study and only the *ABCB1 G2677T* polymorphism was associated with the clinical improvement (98). Noteworthy, the majority of the pharmacogenetic studies with SSRIs did not find an association between the *CYP2D6* genotype and the clinical outcomes of antidepressant drugs (efficacy and adverse effects) (174,216).

I.1.3.2.2.5.2 PAROXETINE

As for the other antidepressant drugs, for PAR the pharmacogenetics research at the level of CYP isoenzymes has been essentially focused on the genetic factors related to CYP2D6. Several works have reported associations between the number of *CYP2D6* active

alleles and PAR metabolism and concentrations (201,204,217). Accordingly, clinical dosing recommendations have been proposed for PAR based on CYP2D6 gPH (177,180,199). Multiple studies have demonstrated that CYP2D6 gUMs have low or undetectable PAR plasma concentrations when compared to CYP2D6 gEMs (201,207–209). Nonetheless, there are no enough data to calculate an initial PAR dose for CYP2D6 gUMs. Thus, as low or undetectable PAR concentrations may be a risk factor for poor therapeutic response or therapy failure, it has been strongly recommended to change to an alternative SSRI not extensively metabolized by CYP2D6 in CYP2D6 gUM patients (Table I.3.3) (177,180,199). However, the clinical impact of this lower PAR exposure was not demonstrated yet (207).

Regarding CYP2D6 gIMs literature is more difficult to evaluate. CYP2D6 diplotypes have been inconsistently categorized as gEMs or gIMs and, consequently, there is no consistent evidence, neither therapeutic recommendations at this level. Nevertheless, CYP2D6 gIMs may have a modest increase in PAR exposure and may be more susceptible to CYP2D6 inhibition by this drug (180). For example, significantly higher PAR concentrations were observed in gIMs patients (one functional allele) compared to gEMs (two functional alleles) or gPMs (no functional allele) in Japanese psychiatric patients (217). Contrarily, other authors did not find differences in the steady-state pharmacokinetics of PAR between gIMs and gEMs (207,218). One possible explanation for these findings is the fact that PAR is metabolised by different CYP isoenzymes, which were not evaluated together.

Lastly, CYP2D6 gPMs have displayed significantly higher PAR plasma exposure when compared to gEMs, which constitutes a risk factor for adverse effects (201,204). Thus, for CYP2D6 gPMs patients it has been recommended to select an alternative SSRI not extensively metabolized by CYP2D6 or to consider a 50 % reduction of recommended starting dose and dose adjustments should be titrated according to response, in order to potentially prevent adverse effects (Table I.3.3). Nonetheless, the level of evidence supporting these recommendations for CYP2D6 gPMs is low, because there are limited data describing dose–concentration and concentrations-clinical outcomes relationships of PAR, as well as the impact of these genetic factors on the corresponding clinical outcomes (180).

I.1.3.2.2.5.3 VENLAFAXINE

VEN is not the mostly studied drug among the three antidepressants FLU, PAR and VEN; however, it is the drug with the most consensual findings across studies, particularly at the level of the impact of genetic factors related to CYP2D6, CYP2C9 and CYP2C19 on the drug outcomes. Clinical data demonstrate that the pharmacokinetics and clinical outcomes of VEN are affected by the functional activity of the polymorphic CYP2D6 enzyme (68). In agreement, the main metabolic route of VEN (*O*-demethylation to ODV) and VEN and ODV concentrations have been found to be strongly dependent of CYP2D6 genotype/gPH/phenotype with apparent stereoselectivity toward the *R*-venlafaxine (R-VEN) enantiomer (117,134,153,154,193,210–213). In turn, CYP2D6 PMs and UMs (gPH or phenotyping determined) have been associated with poor clinical outcomes: specifically CYP2D6 PMs have been associated with an increased risk of adverse effects (117,152,164,165,188,210,214,219–221) and poor therapeutic response (101,131,222,223), while CYP2D6 UMs have been associated with poor therapeutic response (159,214) (Table I.3.3).

Specifically, Lessard *et al.* found higher concentration levels of VEN and lower concentration levels of ODV (major metabolite) for CYP2D6 PMs, including those genotypically CYP2D6 EMs but phenoconverted towards PMs by co-administration of quinidine (a selective CYP2D6 inhibitor); in addition, the PM phenotype (gPH or phenotyping determined) was associated with increased cardiovascular toxicity (210). Another research work found that the oral clearance of *R*-VEN was 9-fold higher in CYP2D6 EMs than in PMs, while the clearance for *S*-venlafaxine (*S*-VEN) was only 2-fold higher in CYP2D6 EMs (gPH or phenotyping determined). Moreover, the co-administration of quinidine to CYP2D6 gEMs resulted in an almost complete inhibition of the metabolic clearance of *R*-VEN, having been verified a 7-fold decrease for *S*-VEN (211). These findings highlight the marked stereoselectivity of the *O*-demethylation reaction catalysed by CYP2D6 toward the *R*-enantiomer (152). In turn, in a study performed in healthy Japanese subjects, *CYP2D6* gIMs (homozygous for **10* allele, a decreased activity allele) had a 4.5-fold higher VEN plasma exposure than gEMs (154). Whyte *et al.* also observed that the plasma concentrations of VEN were significantly higher and those of ODV were significantly lower in patients carrying one or more **4* variant alleles for the *CYP2D6* gene

(gIMs or gPMs) compared to the wild-type gPH (gEMs). This study did not find an association between *CYP2D6* genotype and adverse effects, possibly because the CYP2D6 gPMs were underrepresented (only 3 patients carried *CYP2D6*4/*4* genotype) (212). The impact of the *O*-demethylation phenotype of VEN on its pharmacokinetics and clinical outcome was also assessed by Shams *et al.*, where the authors concluded that the *O*-demethylation phenotype determined by ODV/VEN ratio is strongly dependent of the *CYP2D6* genotype; patients with ratios below 0.3 were all identified as CYP2D6 gPMs [genotypes (**6/*6, *6/*4 or *5/*4*)], while individuals with ratios above 5.2 were all considered gUMs [genotypes *(2x*1)/*1*)]. Moreover, it was also found a higher risk of gastrointestinal adverse effects and hyponatraemia in CYP2D6 PMs (gPH or phenotyping determined) patients (117). A study that evaluated the association of *CYP2D6* genotype and the dose-related effects of VEN demonstrated that patients who lacked a fully active *CYP2D6* allele (gPMs) were not able to tolerate a maintenance dosage higher than 75 mg/day when compared to patients with at least one fully active *CYP2D6* allele (gIMs or gEMs) (219). In the same way, other research works suggested that a CYP2D6 PM status [gPH or phenotyping determined] increases the risk of developing side effects (152,164,165,188,214,220,221). Nevertheless, as currently there are not enough data to allow estimation of dose adjustments, it is recommended selecting an alternative drug or adjusting dose to clinical response and monitoring VEN and ODV plasma concentrations in CYP2D6 PMs (gPH or phenotyping determined). The same recommendations are made for CYP2D6 IMs (177,215) (Table I.3.3).

Furthermore, other studies have also shown that CYP2D6 EMs (gPH or phenotyping) determined] experienced a better clinical response to VEN than PMs; these findings suggest that the metabolism of VEN into ODV is a determinant factor for the clinical outcome (68,101,131,162,222,223). Perhaps in CYP2D6 PMs the alternative metabolic pathway of VEN (*N*-demethylation mediated through CYP3A4, CYP2C9 and CYP2C19) may assume greater importance, which yields pharmacologically inactive or less active metabolites. Consistent with this hypothesis, a study in steady-state conditions reported that the plasma concentrations of NDV were 5.5-fold higher in CYP2D6 gIMs and 22-fold higher in CYP2D6 gPMs than in gEMs (134). Additionally, it has been suggested that CYP2D6 may have an indirect effect on neurophysiologic functioning through its involvement in the generation of serotonin (68,165).

On the other hand, the CYP2D6 UM phenotype (gPH or phenotyping determined) has been associated with a decline in the clinical effectiveness of VEN. This reduced efficacy has been described in patients who did not achieve the VEN plasma concentrations required to produce the desired therapeutic effects, even under treatment with the usually employed effective doses (137,224). However, as this relationship was not observed in other studies and because the patients with CYP2D6 UM phenotype were underrepresented in the population sample, careful conclusions should be drawn from these results (159,214). At this level, it has been recommend to be alert to decreased VEN and increased ODV plasma concentrations, and to titrate the dose to a maximum of 150 % of the normal dose or select an alternative drug in CYP2D6 UMs (gPH or phenotyping determined) (Table I.3.3) (177,215).

To end, few studies have investigated the impact of genotype/phenotype of the CYP2C9 and CYP2C19 on the pharmacokinetics and pharmacodynamics of VEN (137,154,159,214). McAlpine and colleagues investigated the associations between the blood concentrations of VEN and its metabolite ODV, and the genetic polymorphisms of CYP2D6 and CYP2C19 isoenzymes in human subjects. These authors concluded that CYP2C19 catalyses both *O*- and *N*-demethylation reactions in humans, but the presence of genetic variants of the CYP2C19 isoenzyme was not considerably associated with the concentrations found for ODV. The lack of association between CYP2C19 genetic variants and ODV concentrations may possibly be the result of the involvement of CYP2C19 in other metabolic pathways leading to the formation (from VEN) and metabolism (to DDV) of ODV. On the other hand, CYP2C19 genotypes showed a strong association with the concentration levels of total active moiety (VEN + ODV); a fact explained by the involvement of CYP2C19 isoenzyme in conversion of VEN to NDV, as well as in the conversion of ODV to DDV (137).

I.1.3.3 NON-GENETIC FACTORS

Apart from genetic factors, non-genetic factors modulate pharmacokinetics and pharmacodynamics as well. Indeed, genetics is only a part of the drug phenotype and multiple other non-genetic factors co-interact with genetic factors to define the real phenotype. Non-genetic factors may change the gPH, which has been conceptualized as

phenoconversion and, therefore, genotype alone may not be a reliable therapeutic biomarker (68,69,166–170,225,87,95,123,147,160,163–165). Thus, the investigation of the clinical impact of these non-genetic factors on the drug outcomes must be part of the pharmacogenetics and personalized medicine research, including for FLU, PAR and VEN. Overall, non-genetic factors can be divided into two main categories: subjects' intrinsic factors, those related to physiological and pathophysiological factors, and subjects' extrinsic factors, those referred as environmental factors, such as co-medication (68,167–170,176,225–227).

I.1.3.3.1 SUBJECTS' INTRINSIC FACTORS

Multiple subjects' non-genetic intrinsic factors may affect the pharmacokinetics and/or pharmacodynamics of antidepressant drugs, namely age, gender, co-morbidities and depression phenotype.

Regarding age and gender, these may influence both pharmacokinetics and pharmacodynamics. Both factors determine physiological, pathophysiological and environmental differences, namely in terms of body composition, gastric acid production, gastric emptying, plasma proteins, enzyme activity, drug transport, clearance rates, and co-morbid and co-medicated status, which may impact drug pharmacokinetics. Moreover, age and gender also affect the individual neurobiological, psychosocial and humoral status and, hence, personality and behaviour (228).

Specifically, pharmacokinetics of FLU does not appear to be affected by aging (80). Contrarily, increased plasma concentrations of PAR occur in elderly subjects, but the range of concentrations overlaps with those observed in younger subjects and, thereby, no dose adjustments are recommended (81). In turn, a retrospective evaluation of 478 therapeutic drug monitoring analyses of VEN found that patients older than 60 years had about 46 % higher dose-corrected serum levels of VEN and ODV than the younger ones (68). Independently of the age effect on the pharmacokinetics of antidepressant drugs, caution has been advised in the treatment of elderly patients, due to the aging induced potential for renal and hepatic impairment and for changes in neurotransmitter sensitivity and affinity. In these cases, the lowest effective dose should always be used, and patients should be carefully monitored when an increase in the dose is required.

On the other hand, age has been suggested as a moderator of antidepressant therapeutic response, together with gender and menopausal status (225,229). A pooled analysis of eight randomized clinical studies in patients with major depressive disorder and treated with VEN and SSRIs (namely FLU and PAR) found that age, gender, and hormone replacement therapy modulate the antidepressant therapeutic response (229). Whilst VEN therapeutic response was not affected by age, sex, or hormone replacement therapy use, poorer therapeutic response to SSRIs was found in women with age > 50 years (but not in men). Such differences appeared to be eliminated by hormone replacement therapy, which possibly enhances the SSRI outcomes in this group (229). Indeed, other works have suggested age-related variation in the response of women to several types of antidepressants, as well as the potential improvement under hormone replacement therapy. For instance, younger women appeared to respond better to MAOIs and SSRIs, whereas men and older women appeared to respond better to TCAs (225,228). In agreement, premenopausal women showed greater response to the SSRI sertraline than to the TCA imipramine, whereas postmenopausal women responded equally well to the two drugs (225). In another work, the efficacy of FLU in older women was largely restricted to the subgroup of patients taking hormone replacement therapy (229). The main difference between these antidepressant drugs is that SSRIs are strongly serotoninergic, whereas TCAs/MAOIs although have a serotoninergic effect they are essentially noradrenergic agents. Of note, circulating oestrogen levels may modulate central serotoninergic pathways and enhance the serotonin function. This explains why premenopausal women may present a better therapeutic response to SSRIs than older women. Moreover, these results suggest that the antidepressant effects on noradrenergic neurotransmission may have relatively greater importance after menopause, especially if the depressed woman is not taking hormone replacement therapy (229).

Gender differences related to antidepressant adverse effects have been scarcely described in literature. Deleterious effects on sexual drive and satisfaction and weigh gain have been reported in women taking SSRIs. On the other hand, PAR induced sexual dysfunction was found to be more prevalent in men than in women. Further research needs to be done in this area to determine whether true sex differences exist (228,230).

Besides age and gender, pathophysiological and co-morbid status may significantly affect the antidepressant outcomes. As antidepressant drugs, including FLU,

PAR and VEN, are predominantly eliminated by hepatic and renal routes, the most classic clinical situation is the effect of renal or hepatic impairment on the drug pharmacokinetics. These clinical conditions may lead to drug accumulation and a higher predisposition to concentration-dependent toxicity. In detail, hepatic impairment increased the plasma concentrations of FLU and NFLU, whereas renal impairment demonstrated no clinically relevant impact on the pharmacokinetics of FLU and NFLU. Consequently, a lower or less frequent dose of FLU is recommended in patients with hepatic impairment (80). Regarding PAR, both patients with severe renal impairment and those with hepatic impairment displayed increased PAR plasma concentrations and, therefore, the dose should be decreased (81). Similarly, clearance of VEN is decreased by hepatic and renal impairment (127,129,130,133,231). In patients with hepatic cirrhosis, the VEN and ODV half-lives were prolonged by approximately 30 % and 60 % respectively, and their plasma clearances decreased by 50 % and 30 % respectively, when compared to individuals with normal hepatic function (130). In patients with renal impairment, the half-life of VEN was prolonged by approximately 50 % and the plasma clearance was reduced by approximately 24 % (130). Consequently, the dose of VEN should be reduced by approximately 50 % in patients with mild to moderate hepatic impairment or with severe renal impairment, and even more than 50 % in cases of severe hepatic impairment (127,129).

Another challenge for the treatment of depression with antidepressant drugs is the presence of other co-morbidities. The large Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study showed that over 50 % of patients with major depressive disorder had a co-morbid medical condition. Depression commonly co-occur with endocrine conditions (e.g. hypothyroidism, diabetes and obesity), central nervous system disorders (e.g. anxiety, psychotic disorders, Parkinson's disease, multiple sclerosis) and inflammatory conditions (e.g. arthritis) (232). This deserves special attention because patients with depression and other medical disorders have been associated with poor clinical outcomes, namely lower recovery rates, poorer function and higher rates of relapse than patients with just depression (232). For example, thyroid dysfunction is a frequent co-morbidity of depression and subclinical hypothyroidism has been associated with antidepressant therapeutic failure. For that reason, antidepressant treatments have been frequently augmented with thyroid hormones (232). Also, chronic pain conditions

(e.g. arthritis, musculoskeletal conditions and migraine) negatively affect the duration, severity and recurrence of depressive episodes and have been associated with an increased probability of poor therapeutic response to antidepressant drugs (232). In turn, patients with anxiety disorders have presented significantly lower remission rates than those without anxiety. Overall, psychiatric co-morbidities have been associated with worse antidepressant therapeutic response (225). Noteworthy are the inflammatory diseases, once they may have implications both on pharmacokinetics and on pharmacodynamics of antidepressant drugs. First, non-clinical and clinical evidence has supported a phenoconversion and a down-regulation effect of inflammatory conditions associated with elevated pro-inflammatory cytokines (e.g. human immunodeficiency virus infection, cancer and liver disease) on the drug metabolizing CYP isoenzymes, namely CYP2C9, CYP2C19 and CYP2D6 (167–169,176). Then, inflammatory disorders and high levels of cytokines have been associated with poor antidepressant therapeutic response. In fact, one of the basis of the pathophysiology of depressive disorders is a pro-inflammatory state (see section I.1.4.5) (39). On this matter, SSRIs seem to down-regulate pro-inflammatory processes, while anti-inflammatory treatments appear to enhance the efficacy of SSRIs. Nevertheless, the impact of inflammation on the clinical outcomes of antidepressant drugs has been neglected (170).

Lastly, depression phenotype and the clinical features of the disease, such as subtype, age at onset, chronicity and severity have also demonstrated to be moderators of the clinical outcomes of antidepressant drugs. For example, a classic moderator is melancholic *versus* atypical depression. Patients with melancholic depression are more likely to respond to TCAs than MAOIs or SSRIs. On the other hand, patients with atypical depression may respond better to MAOIs than to TCAs. Patients with no atypical depression appear to respond better to TCAs than those with atypical depression. In turn, patients with early onset and chronic depression have presented much lower response rates with TCAs than those with late-onset and non-chronic depression (225).

I. 1.3.3.2 SUBJECTS' EXTRINSIC FACTORS

Co-medication is one of the most important subjects' extrinsic factors with potential to modulate the antidepressant drug outcomes. The presence of multiple drug

therapy (polypharmacy) is common in the real-world setting of treatment of depression. As mentioned above, depressive patients are frequently in a co-morbid state and, therefore, antidepressant drugs are often co-administered with other drugs used to treat concomitant psychiatric, neurologic or somatic disorders. Consequently, polypharmacy carries an increased risk for drug-induced phenoconversion and drug-drug interactions, which may modify the drug-gene interaction and the pharmacokinetics and pharmacodynamics of antidepressant drugs (95,127,233). Indeed, depression is commonly a chronic condition and requires extended periods of treatment. Hence, the possibility of co-administration of additional medications is high. Also, the increased prevalence of depression in elderly patients leads to the antidepressant drugs are used in complex polytherapy regimens. Furthermore, agents such as lithium, atypical antipsychotics, thyroid hormones and even a second antidepressant (e.g. bupropion) have been used to augment the antidepressant response in cases of refractory depression (70,158,234,235). Accordingly, Preskorn *et al.* described that CYP2D6 drug-induced phenoconversion is common in patients being treated for depression. They highlighted that personalized medicine based solely on genetics may be misleading and, for that reason, drug-induced inter-individual variability needs to be considered as well (236). In line with Preskorn *et al.*, Gressier and colleagues recently demonstrated that a CYP2D6 composite phenotype, based on genotype and co-medication with CYP2D6 inhibitors, was able to predict the therapeutic response to CYP2D6 substrate antidepressants (227).

Phenoconversion has been described as a phenomenon whereby a genotypic IM (gIM) or EM (gEM) is converted into a phenotypic PM, leading to genotype-phenotype mismatch. Although the genotype remains immutable, co-medications or certain co-morbidities may affect a person's metabolic capacity. The resulting high phenotypic variability and the extent of genotype–phenotype mismatch in the gEM and gIM groups mean that many of these gEMs and gIMs would behave pharmacologically as PMs. Therefore, the number of phenotypic PM subjects may be greater than those predicted from genotype (gPH) in many clinical situations and association studies, if only genotypic approaches are used to determine the phenotype (168,176). This phenomenon is now known to be more frequent than has been appreciated hitherto. For example, the CYP2D6 PM phenotype is about seven-times more common due to drug-induced phenoconversion (CYP2D6 inhibitors or substrates) than due to the genetic background. Furthermore, the

incidence of phenotypic CYP2C19 PMs following phenoconversion by inhibitors of CYP2C19 (omeprazole or esomeprazole) was found to be ten-fold higher than that of gPMs in the general white population (176). This phenoconversion concept has been centred on the drug metabolizing enzymes, particularly on the CYP isoenzymes. However, other key pharmacokinetic players, such as drug transporters (e.g. P-gp), may suffer alterations on their genetically programmed functional activity due to non-genetic factors. Drugs may affect both the functional activity (phenotype) of drug-metabolizing enzymes and of drug transporters (inhibition, competition or induction) and, thereby, may induce phenoconversion and drug-drug interactions. A wide variety of drugs from a whole range of pharmacotherapeutic classes and other xenobiotics are known to inhibit, induce or act as competitors (substrates) of drug metabolizing enzymes and transporters, including CYP2D6, CYP2C9, CYP2C19 and P-gp. Nowadays, integrative *in silico* databases compiling these data are available, being tools of utmost importance in the evaluation of the potential of drug-induced phenoconversion and drug-drug interactions (79,237).

Phenoconversion and drug-drug interactions are intimately connected. When a phenomenon of drug-induced phenoconversion affects the pharmacokinetics of a co-administered drug, this phenomenon is clinically recognized as a pharmacokinetic-based drug interaction (168,176). Keeping FLU, PAR and VEN in mind, CYP2C9, CYP2C19, CYP2D6 and P-gp are key players of the pharmacokinetics of these antidepressant drugs and phenoconversion and pharmacokinetic-based drug interactions at this level may naturally result in dramatic pharmacokinetic consequences. Such pharmacokinetic changes may adversely impact the pharmacodynamic profile (safety and/or efficacy), depending on the magnitude of the impact, as well as of other individual factors (168). Several works have described and discussed the clinical relevance of drug-drug interactions involving FLU, PAR and VEN (65,74,78,233). For instance, there are clinically relevant pharmacokinetic risks associated with the combination of VEN and bupropion, because bupropion is a potent inhibitor of CYP2D6, the main enzyme responsible for the metabolism of VEN. Thus, bupropion can substantially raise VEN plasma concentrations, which can result in a variety of dose-dependent serotonergic and noradrenergic adverse effects, ranging from increased anxiety and restlessness to increased blood pressure (70,234,235). Another important and common example is the risk of drug-drug interaction between VEN and FLU. VEN is typically used as an alternative to SSRIs in resistant depression. Thus, during

the switching from FLU to VEN, there is potential for pharmacodynamic and pharmacokinetic-based drug interactions. FLU and its main active metabolite NFLU are well-known inhibitors of the key VEN-metabolizing isoenzymes (CYP2D6 and CYP2C19). In addition, these serotonergic agents present a very long half-life (4–16 days), leading to an increased risk of serotonin syndrome in the process of antidepressant switching (70,158).

I.4 PERSONALIZED MEDICINE, PHARMACOGENETICS AND THERAPEUTIC DRUG MONITORING OF ANTIDEPRESSANT DRUGS

I.1.4 PERSONALIZED MEDICINE, PHARMACOGENETICS AND THERAPEUTIC DRUG MONITORING OF ANTIDEPRESSANT DRUGS

More than half a century after the accidental discovery of the first antidepressant drug, neither medications with a real innovative mechanism of action nor reliable biomarkers to guide the therapeutic interventions are used in the routine clinical practice. Drug treatment of depression is still based on a trial and error approach, both concerning drug choice and dosing regimen. This therapeutic approach contrasts with the high inter-individual variability that has been reported and discussed in this chapter at the level of the drug outcomes with the antidepressant drugs. Consequently, efforts have been concentrated in the investigation of personalized medicine strategies, aiming the improvement of the antidepressant drug treatments based on patients' individual characteristics (147,165,238–240).

"… the right pill at the right time for the right patient" is the promise of personalized medicine. Although in its absolute meaning it seems utopic, relativizing this idea personalized medicine means medicine and pharmacology stratified to specific subpopulations. Personalized medicine refers to the application of patient-specific profiles incorporating genetic and genomic data, as well as clinical and environmental factors, with the perspective of providing more effective treatments individually tailored to a given patient or small patient sub-populations, sharing important genotypical and phenotypical features at the pharmacokinetics and pharmacodynamics level (162,171). Basically, personalize medicine implies the use of relevant predictive biomarkers of the clinical outcomes in favour of better clinical outcomes. Therefore, the investigation, identification and validation of clinically relevant therapeutic biomarkers is the limiting step for the success of the implementation of personalized medicine in the clinical practice. Over the last years, different but possibly complementary strategies have pursued this objective, however, without the expected success. Noteworthy, pharmacogenetics and therapeutic drug monitoring have converged in the investigation of personalized medicine and therapeutic biomarkers for antidepressant drug therapy (65,69,70,87,167,176). In fact, the integrated use of classic therapeutic drug monitoring based on drug concentrations (as a pharmacokinetics phenotyping approach) and the genotyping of predictive biomarkers for drug disposition and drug response (as a

pharmacogenetics approach) has been considered as a promising way to optimize the drug treatments with high inter-individual variability and for which the clinical outcomes are difficult to evaluate, such as antidepressant drugs (167,181). As previously discussed, genetic factors related to P-gp, CYP2C9, CYP2C19 and CYP2D6 have deserved particular attention, once they are key players in the bioavailability and biodisposition of antidepressant drugs, including FLU, PAR and VEN (68,69,180,182,203,87,97,98,159,167,168,174,176). In this scope, clinical guidelines have been developed for antidepressant drugs to guide the therapeutic interventions based on the CYP genotypes (178,180,199). Whilst no sufficient data are available to support recommendations for FLU, specific recommendations have been proposed for VEN and PAR. Nonetheless, such recommendations have not been implemented in the clinical practice, much in part due to the lack of evidence supporting their cost/effectiveness. Despite the influence of CYP genetic variants on the antidepressant pharmacokinetics has been repeatedly demonstrated, the impact of such pharmacokinetic differences on the clinical outcomes of antidepressant drugs (efficacy and adverse effects) has not been consistently proved, possibly because this has not been sufficiently, neither appropriately investigated (162,174).

Pharmacogenetic studies have essentially been focused on genetic factors, often related to a unique gene, investigating binary associations between genotype or gPH and pharmacokinetic and/or pharmacodynamic outcomes. However, human-drug interaction is not a simple gene-drug interaction; instead, it is a complex, multigene and multifactorial one. In a real-world setting, the phenotype of these pharmacokinetic-related proteins is co-influenced not only by genetic, but also by non-genetic modulators, with emphasis on co-medication and co-morbidities (167,168,176). Overall, the influence of non-genetic factors and phenoconversion effects has been neglected (167,168,171,176,226,227). This narrow view of pharmacogenetics has been the major cause of the numerous positive, but often conflicting results and, therefore, of the difficult to identify clinically useful biomarkers for the antidepressant drug therapy.

Thus, more holistic study approaches, considering individual genetic and non-genetic factors together, are needed to translate the pharmacogenetics knowledge into clinical practice (68,167,168,176).

I.5 AIMS OF THIS THESIS

I.1.5 AIMS OF THIS THESIS

Regardless of the large number of currently available antidepressant drugs, pharmacotherapy of depression is a major concern due to the high inter-individual variability in the clinical outcomes achieved with the antidepressant drugs. Although there is increasing evidence supporting that a significant portion of the variability is associated with genetic factors, pharmacogenetics alone has not been able to fully explain such findings and the identification of clinically useful pharmacogenetic biomarkers for antidepressant drugs has not been as successful as it would be expected. The hypothesis that has been appointed to explain these findings is that clinically relevant co-related genetic factors have not been studied together, such as the most relevant genes for antidepressant drug pharmacokinetics (*CYP2C9, CYP2C19, CYP2D6* and *ABCB1*), neither have been studied together with other clinically relevant non-genetic moderators of the drug outcomes, such as co-morbidities and co-medication.

Bearing these facts in mind, the main objective of this doctoral thesis was to carry out a comprehensive pharmacometric evaluation of the clinical impact of genetic polymorphisms of the CYP2C9, CYP2C19, CYP2D6 and P-gp and of non-genetic factors, with emphasis on co-morbidities and co-medication, on the pharmacokinetics and pharmacodynamics of widely used antidepressant drugs (FLU, PAR and VEN), aiming at identifying clinically relevant biomarkers for the treatment of depression with these drugs. For this purpose, a multicentre clinical study was planned and developed in the real-world setting of treatment of depression, exploring an integrated pharmacogenetics and therapeutic drug monitoring approach, the *GnG-PK/PD-AD* study. This study aimed to conclude about the clinical importance of genotyping the *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* genes to optimize the safety/efficacy binominal of the treatment of depression with these antidepressant drugs. Ultimately, the current work is a relevant contribution for the approximation of pharmacogenetics and therapeutic drug monitoring, aiming the identification of therapeutic biomarkers. The fact that antidepressant drugs are highly subject to inter-individual variability and poor clinical outcomes, associated with the fact that the antidepressant drugs in study are widely used and of high therapeutic importance for depression, fully justify this clinical investigation.

Thus, the specific objectives defined for the development of this doctoral work were as follows:

- Development and validation of a high-performance liquid chromatography (HPLC) method coupled to fluorescence detection (FLD), based on microextraction by packed sorbent (MEPS), to simultaneously quantify VEN and its pharmacologically active metabolite ODV in human plasma;
- Development and validation of a MEPS/HPLC-FLD method to quantify FLU, PAR and NFLU in human plasma. The development these two bioanalytical tools would be essential to support the therapeutic drug monitoring approach and the clinical pharmacokinetic analysis to be carried out during the *GnG-PK/PD-AD* study.
- Implementation and management of the *GnG-PK/PD-AD* clinical study in multiple health units of the Centre Region of Portugal, which involved the establishment of collaborations with health teams, belonging to the health units included in the study (medical and nurse teams), and submission of the study to competent Ethics Committees and additional required entities for approval. Then, it will be required a multicentre recruitment of adult depressive outpatients under treatment with FLU, PAR or VEN, in accordance with the study protocol and procedures;
- Quantitative analysis of the drug and/or metabolite concentrations (FLU + NFLU, VEN + ODV and PAR) in the plasma samples obtained from the patients included in the study, applying the previously developed and validated bioanalytical methods;
- Genotyping the *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* genes of the patients included in the study, using methods already implemented.
- Realization of an integrated pharmacometric analysis considering the available genetic, pharmacokinetic and clinical data of the included patients.

At the end of this study, it is expected to provide a comprehensive clinical real-world characterization of depressive patients treated with FLU, PAR and VEN, specifically in terms of pharmacokinetic and pharmacodynamic outcomes and relevant genetic and non-genetic individual factors and to identify potential therapeutic biomarkers for the drugs in study.

CHAPTER II

DRUG BIOANALYSIS

II.1 GENERAL CONSIDERATIONS

II.1 GENERAL CONSIDERATIONS

Therapeutic drug monitoring and clinical pharmacokinetic studies require the availability of bioanalytical methods able to reliably and cost-effectively quantify the concentrations of the drugs and metabolites under study. Accordingly, the laboratorial implementation of quantitative bioanalytical methods was the limiting step for the progress of the next phases of the present doctoral project. In fact, before beginning the clinical phase it was crucial to have available *in house* the required bioanalytical methods, safeguarding the bioanalysis of all samples within the acceptable stability margin of the analytes. For this reason, the first objective of this thesis was the development and validation of bioanalytical methods to quantify the plasma concentrations of the antidepressant drugs in study and its main active metabolites, specifically FLU + NFLU, VEN + ODV and PAR.

At this level, HPLC-based methods constitute the universal approach for the separation and quantification of drugs. Over the years, numerous HPLC methods have been reported for the quantitative analysis of these analytes in human plasma. However, most of them are based on labour intensive, time-consuming and expensive sample preparation techniques and many do not contemplate the simultaneous analysis of the parent drug and its main active metabolite (i.e. FLU + NFLU and VEN + ODV). Thus, keeping these facts in mind and considering that these analytes present physicochemical differences, two new HPLC-FLD bioanalytical methods were developed and validated employing the innovative MEPS sample preparation approach, one for the quantification of VEN plus ODV and another for the quantification of FLU plus NFLU and PAR in human plasma. Both methods were fully validated in a concentration range much wider than the usual therapeutic concentration range of the analytes, following the international accepted guidelines for validation of bioanalytical methods, the respective validation parameters (selectivity, limit of detection, limit of quantification, linearity, accuracy, precision, recovery and stability) and the corresponding acceptance criteria.

This stage of the doctoral project was carried out in the analytical facilities of Health Sciences Research Centre (CICS - Centro de Investigação em Ciências da Saúde) of the University of Beira Interior (UBI). The present chapter will present this work of bioanalytical development, which is included in the following original articles:

- Magalhães P, Alves G, Rodrigues M, LLerena A, Falcão A. First MEPS/HPLC assay for the simultaneous determination of venlafaxine and *O*-desmethylvenlafaxine in human plasma. *Bioanalysis.* 2014;6(22):3025–38;
- Magalhães P, Alves G, Llerena A, Falcão A. Therapeutic Drug Monitoring of Fluoxetine, Norfluoxetine and Paroxetine: A New Tool Based on Microextraction by Packed Sorbent Coupled to Liquid Chromatography. *J Anal Toxicol.* 2017;41(7):631–8.

II.2 FIRST MEPS/HPLC ASSAY FOR THE SIMULTANEOUS DETERMINATION OF VENLAFAXINE AND *O*-DESMETHYLVENLAFAXINE IN HUMAN PLASMA

II.2 FIRST MEPS/HPLC ASSAY FOR THE SIMULTANEOUS DETERMINATION OF VENLAFAXINE AND *O*-DESMETHYLVENLAFAXINE IN HUMAN PLASMA

II.2.1 INTRODUCTION

VEN, chemically designated as 1-[2-dimethylamino-1-(4-methoxyphenyl)-ethyl] cyclohexanol (Figure II.2.1), is a second-generation antidepressant agent belonging to the class of SNRIs (68). Despite the large armamentarium of old and new antidepressant drugs currently available, the desired clinical outcomes have not been successfully achieved in many patients, including in those individuals under VEN therapy (147,159,160). It is also well-recognised today that the inter-individual variability found in antidepressant response is often associated with pharmacogenetic/ pharmacokinetic aspects (147,160). Hence, taking into account that antidepressant therapy and their dosage regimens are mostly guided by a trial and error approach, therapeutic drug monitoring based on drug plasma levels may be a useful tool to optimize therapy (70,196,241). In line with this fact, recently published guidelines recommend the therapeutic drug monitoring of VEN, mainly for dose titration and for special subpopulations or problems solving such as therapeutic inefficacy and/or toxicity (70). At this point, it should be also highlighted that the major and pharmacologically active metabolite of VEN, ODV (Figure II.2.1), is equipotent to the parent drug. Thus, therapeutic drug monitoring of VEN should consider not only the parent compound but also the ODV metabolite (70,196,241).

Indeed, important correlations have been identified between the VEN and ODV plasma levels and the antidepressant response or adverse effects (117,210,241,242). Furthermore, the monitoring of VEN and ODV levels may have particular interest as a phenotyping approach to determine the individual metabolizer status for the highly polymorphic cytochrome P450 2D6 isoenzyme, which is involved in the *O*-demethylation of VEN (151,153,193,196,197). Hence, VEN therapy may be adjusted in a more effective manner if the drug plasma levels to which the patient is exposed are known. Therefore, the development of fast and reliable bioanalytical methods for the simultaneous quantification of VEN and ODV is of the utmost importance (70,196,241).

To date, numerous HPLC methods coupled to different detection systems [FLD (243–245), diode array/ultraviolet (DAD/UV) (246–251), coulometric (252) or mass

spectrometry (253–256)] have been reported in literature for the determination of VEN and ODV in human plasma/serum. However, in those methods, sample preparation has been mostly performed through classic sample extraction procedures such as liquid-liquid extraction (LLE) (243,245–247,249,250,255) and solid-phase extraction (SPE) (244,248,251–254,257).

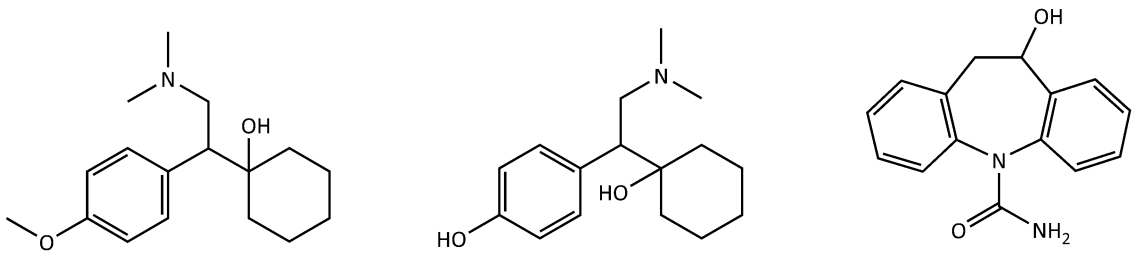
Over the last few years several miniaturized sample preparation techniques have been developed whose importance in bioanalysis is increasingly recognized, among them is the MEPS. This novel sample preparation approach is essentially a miniaturization of the conventional SPE using 1-4 mg of sorbent packed either inside a syringe (100–250 µL) as a plug or between the barrel and the needle as a cartridge. In fact, MEPS has been successfully applied to the quantitative analysis of multiple drugs, namely antibiotics, antihypertensives, antiarrhytmics, antiepileptics, antipsychotics, and even antidepressants (amitriptyline, citalopram, clomipramine, desipramine, doxepin, fluoxetine, imipramine, mirtazapine, nordoxepin, nortriptyline, paroxetine and sertraline) (258). Nevertheless, as far as we know, no bioanalytical assay has been developed for the quantification of VEN and ODV, using MEPS as sample preparation and clean-up methodology.

Hence, the purpose of the present work was to develop and validate the first MEPS/HPLC assay to simultaneously quantify VEN and ODV in human plasma.

II.2.2 MATERIAL AND METHODS

II.2.2.1 MATERIAL AND REAGENTS

Analytical standards of VEN (≥ 98%, lot. 081M4729V) and ODV (≥ 98%, lot. 022M4617V) were purchased from Sigma-Aldrich (St. Louis, MO, USA) as solid hydrochloride salts. Licarbazepine (LIC) was kindly supplied by BIAL (Portela & Ca., SA; S. Mamede do Coronado, Portugal) and it was used as internal standard (IS). The chemical structures of these compounds are shown in Figure II.2.1.



Venlafaxine (VEN) **_O_-desmethylvenlafaxine (ODV)** **Licarbazepine (IS)**

Figure II.2.1 Chemical structures of venlafaxine (VEN), *O*-desmethylvenlafaxine (ODV) and licarbazepine (LIC) used as internal standard (IS).

Acetonitrile (HPLC gradient grade) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and methanol (HPLC gradient grade) from Panreac Química SA (Barcelona, Spain). Ultra-pure water (HPLC grade, >18 MΩ) was obtained through a Milli-Q water apparatus from Millipore (Milford, MA, USA). The remaining reagents used were of analytical grade: sodium di-hydrogen phosphate anhydrous p.a. (Panreac Química SA; Barcelona, Spain), triethylamine (Fisher Scientific; Leicestershire, UK), trichloroacetic acid (Sigma–Aldrich; St. Louis, MO, USA), ammonium hydroxide (24.5%) (J.T.Baker; Deventor, Holland), ethyl acetate (VWR Prolab; Leuven, Belgium) and formic acid (98-100%) (Merck; Darmstadt, Germany). MEPS 250 µL syringe and MEPS BIN (barrel insert and needle) containing ∼4 mg of solid-phase silica – C18 material (SGE Analytical Science, Australia) were purchased from ILC (Porto, Portugal).

II.2.2.2 PLASMA SAMPLES

The blank human plasma used for preparing the calibration standards and quality control (QC) samples was obtained from healthy blood donors with the collaboration of the Portuguese Blood Institute. The authentic plasma samples used to demonstrate the clinical application of the method were collected from outpatients treated at Extremadura Health Care Services (SES, Spain). All plasma samples were only obtained after the written informed consent form to be signed by each subject and were performed according to the ethical principles of the Declaration of Helsinki.

II.2.2.3 STOCK SOLUTIONS, STANDARDS AND QC SAMPLES

Stock solutions of VEN, ODV, and LIC (IS) were separately prepared in methanol at 1 mg/mL. From the stock solutions were also individually prepared intermediate solutions of VEN and ODV at 50 µg/mL and 5 µg/mL by dilution with methanol. Afterwards, appropriate volumes of the intermediate solutions were mixed and diluted with ultra-pure water/methanol (75:25, v/v) to afford six combined spiking solutions at 0.05, 0.1, 0.3, 1, 3 and 5 µg/mL for VEN and at 0.1, 0.2, 0.4, 1, 3 and 5 µg/mL for ODV. These combined solutions were daily used for spiking blank plasma in order to obtain six different standards covering the calibration curve ranges of 10-1000 ng/mL for VEN (10, 20, 60, 200, 600 and 1000 ng/mL) and 20-1000 ng/mL for ODV (20, 40, 80, 200, 600 and 1000 ng/mL). The working solution of IS was daily prepared in water–methanol (75:25, v/v) at 200 µg/mL by means of a suitable dilution of the corresponding stock solution. The intermediate and working solutions were stored at 4 ºC in the dark, and the stock solutions were stored at -80 ºC.

QC samples were independently prepared in the same matrix (blank human plasma) at low (QC_1: 30 ng/mL for VEN and 60 ng/mL for ODV), middle (QC_2: 500 ng/mL) and high (QC_3: 900 ng/mL) concentrations representative of the range of calibration curves. Two other QC samples were also prepared: one at the concentration of the lower limit of quantification (LLOQ; QC_{LLOQ}: 10 ng/mL for VEN and 20 ng/mL for ODV) and the other at a concentration level above the upper limit of quantification (ULOQ; QC_{dil}: 5000 ng/mL) in order to evaluate the dilution integrity of samples.

II.2.2.4 CHROMATOGRAPHIC EQUIPMENT AND CONDITIONS

The instrumental analysis was carried out through an ultra-high-performance liquid chromatography (UHPLC) system (Agilent 1290 Infinity Binary LC system) coupled with a FLD detector (Agilent 1260 Infinity; G1321B). All control of the instrumental parts and data acquisition were achieved by *Agilent ChemStation* software (Agilent Technologies).

The separation of VEN, ODV and IS was reached in less than 6 min using a mobile phase of 10 mM phosphate buffer with 0.25% of triethylamine (pH 3.3)/acetonitrile

(83:17, v/v) pumped isocratically at a flow-rate of 1 mL/min on a reversed-phase LiChroCART® Purospher Star-C_{18} column (55 mm × 4 mm; 3 µm particle size) purchased from Merck KGaA (Darmstadt, Germany). The column was thermostated at 45 °C. The mobile phase was filtered through a 0.2 µm filter and degassed ultrasonically for 15 min before use. The injection volume was 20 µL and the excitation and emission wavelengths were set at 233 and 315 nm, respectively.

II.2.2.5 SAMPLE PREPARATION AND MEPS EXTRACTION

To each plasma sample (100 µL) was added 20 µL (4 µg) of the IS working solution, 60 µL of ultra-pure water and 20 µL of 20% trichloroacetic acid aqueous solution. This mixture was vortex-mixed for 30 s and centrifuged at 13,000 rpm for 3 min at room temperature and the supernatant was transferred to a clean vial. Afterwards, the pellet was resuspended with 50 µL of ultra-pure water and 2 µL of 20% trichloroacetic acid aqueous solution and the mixture was again vortex-mixed and centrifuged using the aforementioned conditions. The resulting supernatant was mixed with that previously obtained and the final supernatant volume was then subjected to the MEPS procedure.

All steps of the MEPS procedure were manually carried out (off-line). Before being used for the first time, the MEPS cartridge was activated with 3 × 200 µL of methanol and then conditioned with 2 × 200 µL of ultra-pure water. Afterwards, the supernatant mixture of the pre-treated sample was drawn into the MEPS syringe and discharged in the same vial for three times (cycles) at a flow rate of approximately 5 µL/s. The MEPS cartridge was washed with 50 µL of water to remove interfering substances, and then for the elution of analytes was used 200 µL of methanol. Then, the methanolic extract was evaporated to dryness under a gentle stream of nitrogen at 45 ºC, redissolved in 100 µL of ultra-pure water/acetonitrile (83:17, v/v) and injected (20 µL) into the chromatographic system. After each extraction, in order to avoid memory effects, the MEPS sorbent was successively washed/reconditioned with 20 × 200 µL of methanol and 2 × 200 µL of ultra-pure water. This step not only prevents carryover effects, but also allows the conditioning of the sorbent for the next extraction. Each MEPS device was re-used for about 200 extractions before it was discarded.

II.2.2.6 METHOD VALIDATION

This method was validated in accordance with the internationally published guidelines (259,260).

Selectivity of the method was assessed by evaluating potential interference from endogenous compounds and other commonly co-administered drugs at the retention times of the analytes (VEN and ODV) and IS. In order to guarantee the absence of endogenous interferences (matrix effects), six blank human plasma samples from different individuals were analysed. Interference from other commonly co-prescribed drugs such as acetylsalicylic acid, alprazolam, amiloride, amitriptyline, caffeine, chlorpromazine, cimetidine, citalopram, clomipramine, clorazepate, clozapine, diazepam, diltiazem, dipyridamole, dosulepine, droperidol, duloxetine, escitalopram, flecainide, fluoxetine, fluvoxamine, haloperidol, hydroxyzine, ibuprofen, lamotrigine, levopromazine, maprotiline, mexazolam, mianserine, mirtazapine, nifedipine, nimesulide, nortriptyline, olanzapine, omeprazole, oxazepam, paracetamol, paroxetine, phenobarbital, promazine, promethazine, protriptyline, quetiapine, risperidone, selegiline, sertraline, trazodone, verapamil, warfarin and zolpidem was also tested by injecting solutions of these compounds at 10 µg/mL.

To assess the linearity of the method, calibration curves were constructed with six calibration standards covering the range of 10-1000 ng/mL for VEN and 20-1000 ng/mL for ODV and assayed on five distinct days (n = 5). Along with each set of calibration standards, a blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS) were also analyzed. The calibration curves were obtained by plotting analytes (VEN or ODV)/IS peak area ratios against the corresponding nominal plasma concentrations. The data were subjected to a weighted linear regression analysis (261).

The LLOQs were defined as the lowest concentration levels that could be quantified with acceptable precision [expressed as a coefficient of variation (CV) value lower than or equal to 20%] and accuracy [expressed as a deviation from nominal concentration (*bias*) within ±20%] by analysing plasma samples in replicates (n = 5). The limits of detection (LODs) were determined by a signal-to-noise approach and were established as the concentrations that yields a signal/noise ratio of 3/1.

Inter-day precision and accuracy were evaluated by means of QC samples (QC_{LLOQ}, QC_1, QC_2, QC_3 and QC_{dil}) analyzed in five days (*n* = 5), whereas the intra-day precision and accuracy data were acquired by analysing five sets of QC samples in a single day (n = 5). The QC_{dil} sample (5000 ng/mL) was considered in order to assess the precision and accuracy of the dilution integrity of plasma samples; thus, prior to the extraction of each QC_{dil} sample a ten-fold (1/10) dilution step with blank human plasma was performed to obtain concentrations of the analytes (VEN and ODV) at approximately the midpoint of the calibration curves (500 ng/mL). The acceptance criterion for precision was a CV value lower than or equal to 15% (or 20% in the QC_{LLOQ}) and for accuracy was a *bias* value within ±15% (or ±20% in the QC_{LLOQ}).

The recovery of the analytes (VEN and ODV) from plasma was investigated at the three concentrations levels (QC_1, QC_2 and QC_3 samples) by assaying five replicates. The absolute recovery of the analytes was calculated by comparing the peak area of the analytes from extracted QC samples (*n* = 5) with the corresponding peak area obtained from the direct injection of non-extracted aqueous/acetonitrile solutions (83:17, v/v) at the nominal concentrations (*n* = 5). Similarly, the absolute recovery of the IS was estimated at the concentration level employed during the sample analysis.

Human plasma stability of VEN and ODV was evaluated using QC_1 and QC_3 samples, in replicate (*n* = 5), at room temperature for 4 h, at 4 ºC for 24 h, and at −20 ºC for 30 days, simulating the sample handling and storage conditions prior to analysis. The stability of the target analytes (VEN and ODV) in plasma was also studied after three freeze-thaw cycles at −20 ºC; for that, aliquots of spiked plasma samples (QC_1 and QC_3) were stored at −20 ºC for 24 h, thawed unassisted at room temperature and, when completely thawed, the samples were refrozen for 24 h under the same conditions until completing the three cycles. The post-preparative stability of VEN and ODV was also studied at room temperature for 12 h in processed samples. Stability was assessed by comparing the data of QC samples analysed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples); the acceptance criterion for stability was a stability/reference samples ratio within 85–115% (*n* = 5).

II.2.2.7 CLINICAL APPLICATION

To assess the suitability of the proposed HPLC assay and its validity for clinical application, plasma samples obtained from five adult outpatients under VEN therapy and treated at Extremadura Health Care Services (SES, Spain) were analysed.

II.2.3 RESULTS AND DISCUSSION

II.2.3.1 CHOICE OF THE CHROMATOGRAPHIC AND DETECTION CONDITIONS

Taking into account our previous experience (262–264) and also the published data on bioanalysis of VEN (245,255,257), a reversed-phase LiChroCART® Purospher Star C_{18} column (55 mm × 4 mm; 3 µm particle size) with a short length was selected in order to enable the use of smaller volumes of solvents and shorter run times.

In the preliminary studies, a good chromatographic resolution and a relative fast elution of the analytes (ODV and VEN) were achieved using a mobile phase composed of 10 mM sodium phosphate buffer and acetonitrile (83:17, v/v) isocratically pumped at a flow rate of 1 mL/min. The influence of mobile phase pH was also evaluated and the pH value of 3.3 was selected. For higher pH values the elution of all analytes was delayed increasing the run time. In fact, as both compounds are basic, the use of a more acidic mobile phase can determine an increased ionized form and a lower hydrophobicity; thus, in these conditions weaker hydrophobic interactions are established with the column stationary phase, which leads to a faster elution and a shortening of the run. It is also worth to note, that the column temperature at 45 ºC improved the run time and even the peak resolution, without significant impact on the chromatographic separation (119,253,265,266). Moreover, triethylamine was added to the mobile phase to suppress the peak tailing effect (267).

Regarding the FLD conditions, different excitation and emission wavelengths were tested in the range of 200–300 nm and 290–450 nm, respectively, having been defined 233 and 315 nm, since they allowed the best compromise between sensitivity for all the analytes and high selectivity against endogenous substances.

Another important aspect to be considered during the development of this bioanalytical method was the selection of the IS; several compounds with FLD properties such as protriptyline, clomipramine, fluoxetine, haloperidol, gatifloxacin and LIC were tested. Among them, LIC was chosen because it displayed the most similar extraction and chromatographic behaviour in relation to the target analytes (VEN and ODV), including good FLD detection proprieties at the wavelengths defined, a suitable retention time with a good chromatographic resolution and a relatively high extraction efficiency. Moreover, LIC is commercially available and, although it is the main metabolite of two antiepileptic drugs (oxcarbazepine and eslicarbazepine acetate), they are not approved to be used in the psychiatry scope as antidepressant or antipsychotic agents.

In summary, the established chromatographic conditions enable the development of a simple and fast (chromatographic run time about 6 min) HPLC-FLD assay that could be easily applied in the majority of clinical laboratories.

II.2.3.2 SAMPLE PREPARATION: OPTIMIZATION OF MEPS CONDITIONS

Within the framework of this research work, the study of the factors affecting the performance of MEPS was considered the critical step.

Consistent with our experience (258,264), Abdel-Rehim (268) has also alerted to the need of some sample pre-treatment steps before sample loading to avoid the rapid clogging of MEPS cartridges. Therefore, in the proposed bioanalytical assay the plasma samples were deproteinized with 20% trichloroacetic acid before MEPS loading. Despite the deproteinization of plasma samples, it should be emphasized that this work never aimed to directly compare protein precipitation and MEPS as sample preparation methodologies. The main goal of the deproteinization step was to prolong the life of MEPS cartridges. This strategy enabled cleaner chromatograms to be obtained, yielding a suitable selectivity to accurately quantify low concentrations of VEN and ODV (few ng/mL), and the same cartridge was reused for about 200 extractions without affecting MEPS performance. It is also true that protein precipitation leads to sample dilution, which can compromise the sensitivity of the method, but such dilution effect is herein counteracted by MEPS procedure.

As VEN and ODV have been previously extracted by means of SPE procedures using C_8 or C_{18} hydrocarbon sorbents (244,257,269,270), in a preliminary set of assays the performance of C_8 and C_{18} MEPS cartridges was compared using saline aqueous solution. C_{18} MEPS cartridges afforded an improved extraction efficiency for all compounds of interest (VEN, ODV and IS) and, therefore, they were chosen and used in the following optimization steps.

Regarding the sorbent conditioning step, there is not a large variability among the published MEPS protocols (258). Thus, bearing in mind that most frequently used conditions involve the sorbent activation through multiple passages of methanol followed by sorbent equilibration with water, in our case the sorbent conditioning with 5 × 200 µL of methanol and 2 × 200 µL of water was defined *a priori*. Furthermore, taking into consideration the available literature (258), unless otherwise referred, the basal conditions in MEPS procedures for the washing and elution steps was 100 µL of water and 100 µL of methanol, respectively.

In the present work the influence of the number of draw-ejected cycles, and the nature and volume of washing and elution solutions in the recovery of the compounds of interest (VEN, ODV and IS) was investigated (Figure II.2.2) in order to obtain the best compromise between selectivity and recovery with no significant carryover effects. At this point, using the aforementioned basal MEPS conditions, each experimental variable was tested in replicate (n = 3) and the results were evaluated based on the mean peak area of each compound of interest (VEN, ODV and IS). All these experiments were carried out in human plasma samples spiked at 500 ng/mL for VEN and ODV and using 20 µL of the IS working solution (200 µg/mL).

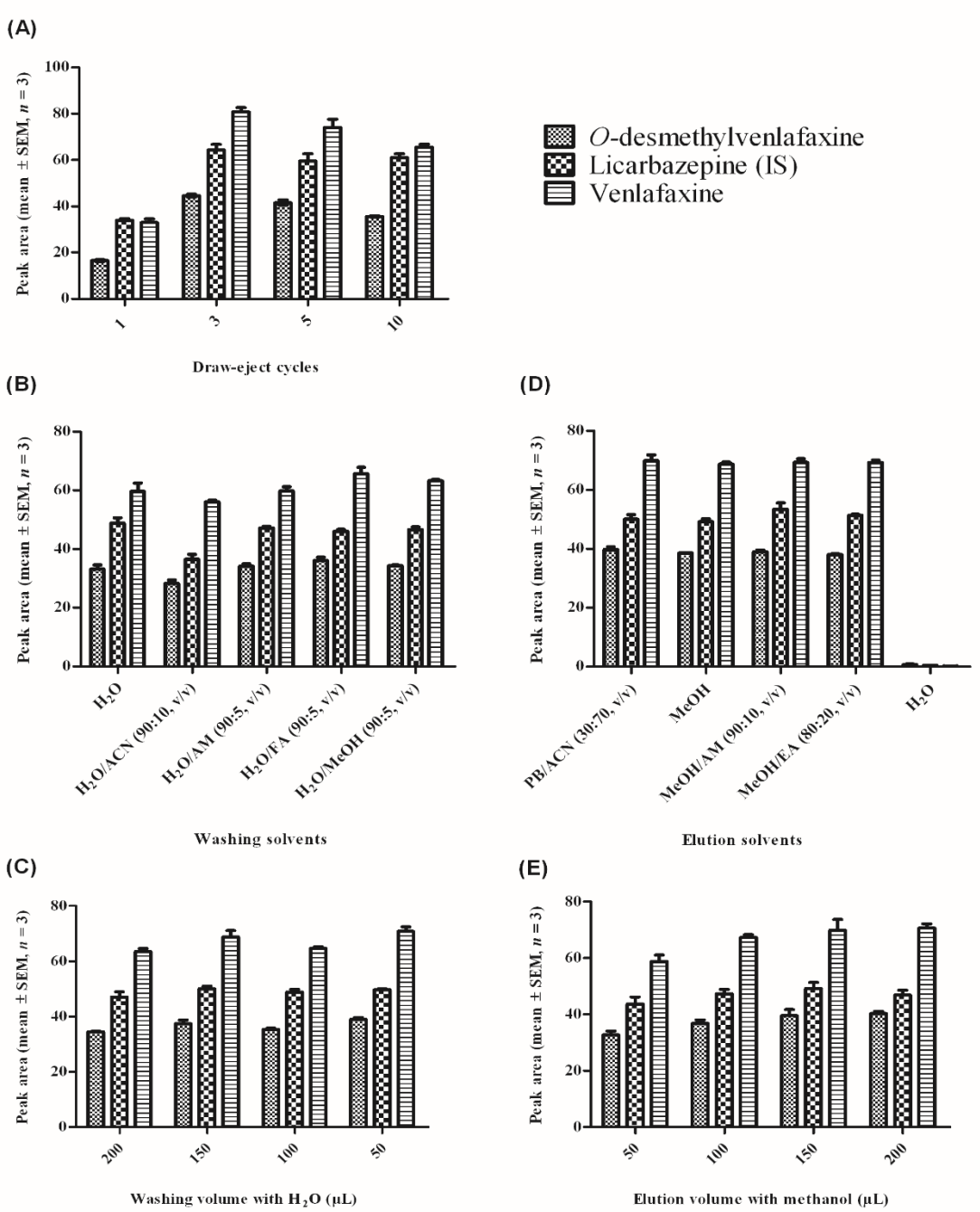


Figure II.2.2 Effect of different Microextraction by packed sorbent (MEPS) conditions on the extraction efficiency of the analytes [*O*-desmethylvenlafaxine (ODV) and venlafaxine (VEN)] and licarbazepine (LIC) used as internal (IS): draw-eject cycles (A), washing solutions (B), washing volume using water as washing solution (C), elution solutions (D), and elution volume using methanol as elution solution (E). All the experiments were carried out with human plasma samples spiked at 500 ng/mL for VEN and ODV and adding 20 µL of IS solution at 200 µg/mL. The error bars on each column correspond to the standard error of the mean (SEM). ACN, acetonitrile; AM, ammonium hydroxide; EA, ethyl acetate; FA, formic acid; MeOH, methanol; PB, Phosphate buffer.

II.2.3.2.1 NUMBER OF LOADING DRAW-EJECT CYCLES

The study of the influence of the number of sample loading cycles (1 to 10) on the signal-response of VEN, ODV and IS showed that the maximum recovery of all compounds was obtained through three draw–eject cycles (Figure II.2.2A); therefore, the number of three sample loading cycles was selected for the MEPS procedure. In this step a low flow-rate (approximately 5 µl/s) was considered in the sample draw-eject cycles, because it has been shown to be a determinant factor to obtain a good percolation between the target compounds and the MEPS sorbent.

II.2.3.2.2 NATURE AND VOLUME OF WASHING AND ELUTION SOLUTIONS

First, considering a fixed volume of 100 µL, the nature of the most appropriate solvent/solution for the washing and elution steps was studied (Figure II.2.2B and 2C). Then, the effect of the volume of washing and elution solutions (200, 150, 100 and 50 µL) was examined (Figure II.2.2D and 2E). The choice of the tested solvents/solutions was based on previous experiments for VEN and ODV using SPE, as well as in the washing and elution solutions usually employed in MEPS procedures, considering the chemical properties of the target compounds and the hydrophobic interactions as the main mechanism subjacent to its retention in a C_{18} sorbent (257,258).

The purpose of the washing step is to selectively remove the interferences from the MEPS sorbent without significant loss of the compounds of interest. Hence, the cleaning of chromatograms from extracted blank plasma samples was also taken into account. In this research work, pure water and four aqueous solutions (100 µL) with low percentages of different organic solvents were assessed as washing agents; under these different washing conditions a great similarity was unexpectedly found in terms of selectivity and extraction yields, which led to the selection of water as the washing solvent, especially due to practical reasons (Figure II.2.2B). A better absolute recovery was achieved using a washing volume of 50 µL of water (Figure II.2.2C).

On the other hand, the elution efficiency of VEN, ODV and IS from C_{18} MEPS cartridges was firstly tested using 100 µL of different elution solvents/solutions with distinct polarity indexes; the better results in terms of compound recovery, selectivity and

practicability led us to choose methanol as the elution solvent (Figure II.2.2D). For instance, the elution with phosphate buffer (pH 3.3)/acetonitrile (30:70, v/v) and methanol/ammonium hydroxide (90:10, v/v) solutions resulted in the appearance of interferences at the retention time of VEN. Thus, overall, the pH change in the elution step is not advantageous. Additionally, in the present study the influence of the elution methanol volume was investigated in the range 50–200 µL for optimizing the amount extracted (Figure II.2.2E). Although only a slight increase in the mean signal-response for the target analytes (VEN and ODV) had been found in the range 100–200 µL of methanol, the higher elution volume (200 µL of methanol) was selected in order to minimize the possible variability in the extraction efficiency at this critical step of the MEPS procedure.

II.2.3.2.3 CARRYOVER EFFECTS

The carryover was also carefully investigated to ensure the total removal of the analytes and endogenous compounds from the MEPS sorbent before the extraction of the next sample. For that, the carryover effects were tested after extraction and analysis of consecutive plasma samples spiked at the highest standard concentration (1000 ng/mL) followed by extraction of blank plasma samples. According to the obtained data, to avoid significant memory effects, the MEPS sorbent needs to be sequentially cleaned/reconditioned with 20 x 200 µL of methanol and 2 × 200 µL of water after each extraction.

II.2.3.3 METHOD VALIDATION

II.2.3.3.1 SELECTIVITY

Blank plasma samples from several individuals were tested (n = 6) and no significant interferences were found at the retention times of VEN, ODV and IS. Representative chromatograms of blank and spiked human plasma samples are provided in Figure II.2.3.

Similarly, none of the numerous tested drugs which can be co-administered with VEN in the clinical practice interfered with the chromatographic peaks.

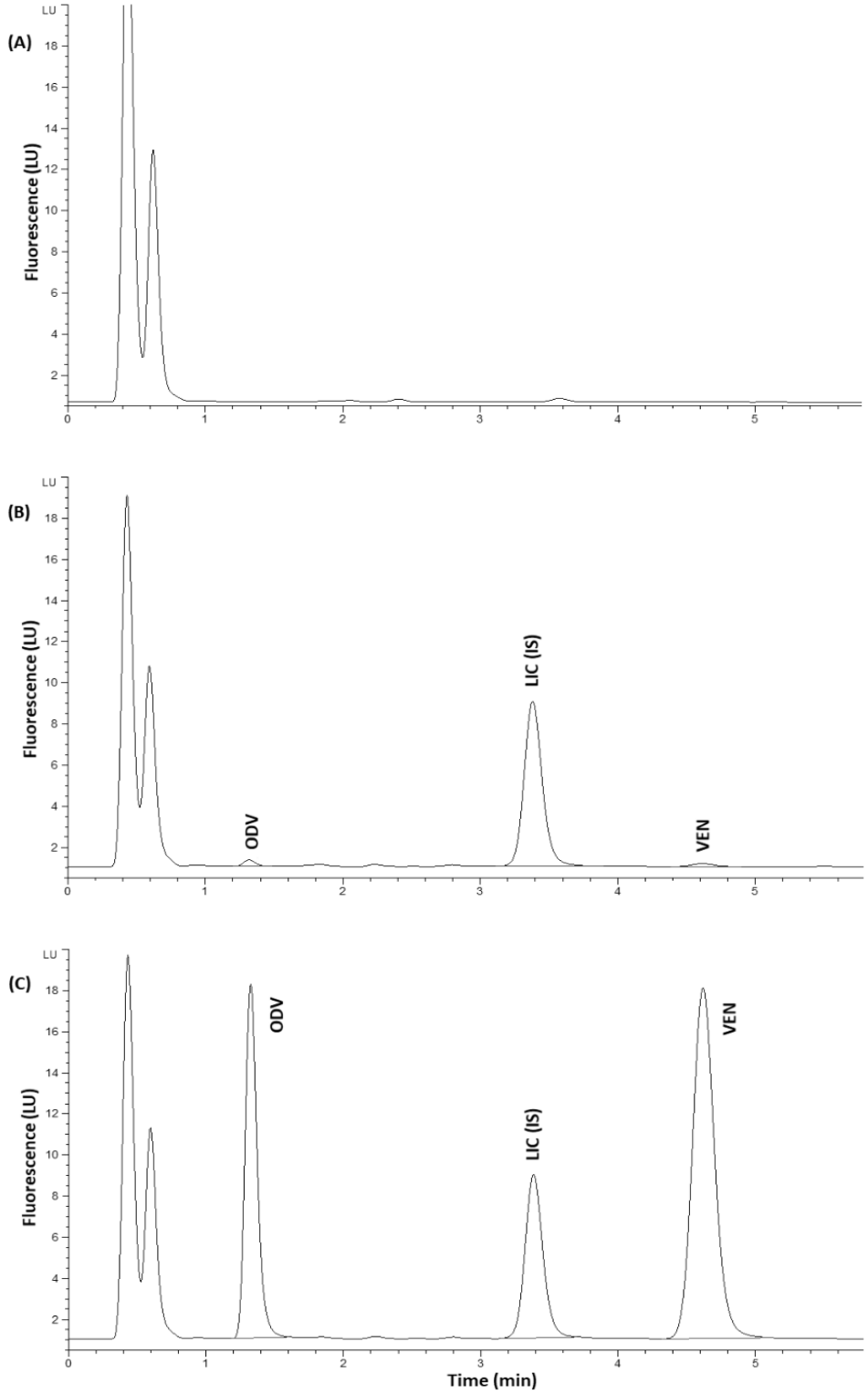


Figure II.2.3 Typical chromatograms of extracted human plasma samples obtained by the MEPS/HPLC-FLD method developed: (a) blank plasma; (b) plasma spiked with internal standard [IS; licarbazepine (LIC)] and the analytes [*O*-desmethylvenlafaxine (ODV) and venlafaxine (VEN)] at concentrations of the lower limit of quantification (20 ng/mL for ODV and 10 ng/mL for VEN), and (c) at concentrations of the upper limit of calibration range (1000 ng/mL).

II.2.3.3.2 LINEARITY, LLOQ AND LOD

The calibration curves were linear (r^2 ≥ 0.9976) over the concentration range of 10-1000 ng/mL for VEN and 20-1000 ng/mL for ODV. Heteroscedasticity was verified as a result of the wide calibration range established and, consequently, the use of weighted linear regression analysis was considered. The best-fit weighting factor for VEN and ODV was $1/x^2$. Thus, the weighted regression equations (n = 5) of the calibration curves using $1/x^2$ as weighting factor were y = 0.00249 x – 0.00368 (r^2 = 0.9976) for VEN and y = 0.00138 x + 0.00371 (r^2 = 0.9987) for ODV, where y represents the analyte/IS peak area ratio and x represents the plasma concentration.

The LLOQ was experimentally defined as 10 ng/mL for VEN and 20 ng/mL for ODV, with good precision (CV ≤ 5.1%) and accuracy (*bias* values between -7.0 and 8.7%). The LOD was established at 2 ng/mL for VEN and 5 ng/mL for ODV. At this point, it is important to refer that the LLOQs attained in the present assay are very satisfactory, particularly when compared with those obtained by other authors, considering the respective sample volume used. Indeed, although this bioanalytical method requires a smaller volume of sample (100 µL), the LLOQs achieved are, in the most cases, the same or even lower than those verified in several HPLC-DAD/UV (247,248,250,271,272) and HPLC-FLD (245) methods currently published. There are some HPLC-FLD methods with lower values of LLOQs, but in these cases higher volumes of plasma (200-1000 µL) were used (243,244,273).

To conclude, once the quantification ranges of the present technique are much wider than the respective therapeutic window of the compounds (70-300 ng/mL for VEN and 200-500 ng/mL for ODV) (249,257), this method can be applied both in pharmacokinetic, pharmacogenetic/pharmacokinetic and toxicological studies.

II.2.3.3.3 PRECISION AND ACCURACY

The data for intra- and inter-day precision and accuracy are reported in Table II.2.1.

Table II.2.1 Inter- and intra-day precision and accuracy for the determination of venlafaxine (VEN) and *O*-desmethylvenlafaxine (ODV) in human plasma samples at the concentrations of the lower limit of quantification (QC_{LLOQ}), at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges and following the sample dilution by a 10-fold factor (QC_{dil}).

Analyte	Nominal concentration (ng/mL)	Experimental concentration (mean ± SD, ng/mL)	Precision (% CV)	Accuracy (% *bias*)
Inter-day (n = 5)				
VEN	10 (QC_{LLOQ})	10.0 ± 0.4	4.4	-0.5
	30	28.1 ± 1.1	4.2	-6.5
	500	503.7 ± 10.1	2.0	0.8
	900	949.9 ± 17.1	1.8	5.5
	5000 (QC_{dil})	4952.9 ± 157.9	3.2	-0.9
ODV	20 (QC_{LLOQ})	21.8 ± 1.0	5.1	8.7
	60	59.5 ± 1.5	2.6	-0.9
	500	498.1 ± 12.9	2.6	-0.4
	900	931.9 ± 23.4	2.5	3.5
	5000 (QC_{dil})	4985.9 ± 154.2	3.1	-0.3
Intra-day (n = 5)				
VEN	10 (QC_{LLOQ})	9.3 ± 0.1	1.8	-7.0
	30	26.9 ± 0.4	1.7	-10.3
	500	481.1 ± 7.4	1.6	-3.8
	900	949.3 ± 43.8	4.6	5.5
	5000 (QC_{dil})	5214.2 ± 145.5	2.8	4.3
ODV	20 (QC_{LLOQ})	19.7 ± 0.7	4.4	-1.3
	60	57.7 ± 0.7	1.3	-3.8
	500	469.7 ± 7.8	1.7	-6.1
	900	900.1 ± 20.4	2.3	0.0
	5000 (QC_{dil})	5155.4 ± 142.1	2.8	3.1

The intra- and inter-day CV values did not exceed 5.1%, and the intra- and inter-day *bias* values varied between −10.3 and 8.7%. These data are in accordance with the

criteria internationally established and required for the validation of bioanalytical methods. Therefore, the HPLC–FLD method herein developed is reliable, accurate and reproducible over the wide calibration range proposed, including when a 10-fold dilution of plasma samples with blank plasma is necessary. Actually, according to various toxicological studies (188,272,274,275) VEN and ODV concentrations can be higher than 1000 ng/mL (ULOQ) in intoxication cases, being necessary to apply a sample dilution to quantify with accuracy and precision these samples. However, most of VEN's methods reported in literature did not consider the study of the dilution integrity and, therefore, in most cases, these high concentrations were not covered, or a narrower calibration range was defined.

II.2.3.3.4 ABSOLUTE RECOVERY

The mean absolute recoveries for VEN and ODV ranged from 79.5% to 82.5% and 72.7% to 76.8%, respectively and showed very low CV values (Table II.2.2).

Table II.2.2 Absolute recovery (%) of venlafaxine (VEN) and *O*-desmethylvenlafaxine (ODV) from human plasma samples at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges (n = 5).

Analyte	Nominal concentration (ng/mL)	Recovery (%) Mean ± SD	CV (%)
VEN	30	80.7 ± 2.6	3.2
	500	82.5 ± 1.4	1.7
	900	79.5 ± 1.3	1.6
ODV	60	76.0 ± 1.9	2.5
	500	76.8 ± 1.4	1.8
	900	72.7 ± 2.5	3.5

CV, coefficient of variation; SD, standard deviation

On the other hand, the mean absolute recovery for the IS was 70.8%, with a CV of 4.7%. Revising the literature, there are other authors reporting superior recovery values for both analytes (VEN and ODV), but essentially by means of conventional SPE procedures (244,248,257). Thus, the lower extraction efficiencies for VEN and ODV in this

assay may be justified by the fact of MEPS to be a miniaturized version of SPE. Indeed, as the determination of VEN and ODV in plasma samples by means of MEPS has not been yet published in the literature, our results cannot be directly compared with similar methods. However, the extraction efficiency of the described bioanalytical methodology is perfectly framed with previous results achieved with MEPS as sample preparation procedure (258).

II.2.3.3.5 STABILITY

The analytes of interest (VEN and ODV) were stable in human plasma at room temperature for 4 h, at 4 ºC for 24 h, after three freeze-thaw cycles at -20 ºC and at -20 ºC for 30 days. The stability of VEN and ODV was also demonstrated in processed plasma samples at room temperature for 12 h. Considering all the studied conditions, the stability/reference samples ratio varied from 96.6-106.1% and 91.3-103.4% for VEN and 97.4-109.0% and 91.5-104.6% for ODV in QC_1 and QC_3 samples, respectively.

II.2.3.3.6 CLINICAL APPLICATION OF THE METHOD TO REAL PLASMA SAMPLES

The MEPS/HPLC-FLD method herein reported was successfully applied to the quantitative determination of VEN and ODV in plasma samples taken from five adult patients undergoing treatment with VEN. The prescribed VEN regimen for each individual (ID), the co-medication and the respective trough concentrations (C_{min}) obtained are summarized in Table II.2.3 According to the guidelines for therapeutic drug monitoring in psychiatry, all the samples were collected in steady-state conditions, immediately before the administration of the morning dose (70).

Table II.2.3 Plasma concentrations of venlafaxine (VEN) and *O*-desmethylvenlafaxine (ODV) in real human plasma samples obtained from patients taking VEN orally and respective prescribed regimen and other co-medication. Samples were collected in the morning before the next dose (trough concentrations, C_{min}).

Patient	Co-medication	VEN regimen (dose/ day)	Concentration (ng/mL)	
			VEN	ODV
ID_1	Alprazolam, diazepam, gabapentin, olanzapine, paracetamol, fentanyl, pregabalin	150 mg	59.0	121.1
ID_2	Alprazolam, calcium carbonate/colecalciferol, hydroxychloroquine, montelukast, omeprazole, prednisone, pregabalin, sodium risedronate, simvastatin	150 mg	352.7	215.0
ID_3	Quetiapine, clorazepate	75 mg	32.3	48.3
ID_4	Alprazolam, lorazepam, omeprazole	150 mg	10.2	96.8
ID_5	Diosmin, esomeprazole, pancreatin, sulfipiride	75 mg	117.6	58.2

A representative chromatogram (ID_5) of the application of the method to real plasma samples is presented in Figure II.2.4 It is evident that the chromatographic behaviour of the compounds of interest (VEN, ODV and IS) is similar to that was achieved by the analysis of spiked plasma samples. Additionally, no interference from endogenous compounds of the patient plasma samples or from the co-administered drugs (Table II.2.3) were found at the retention time of the analytes (VEN, ODV and IS) in any of the samples.

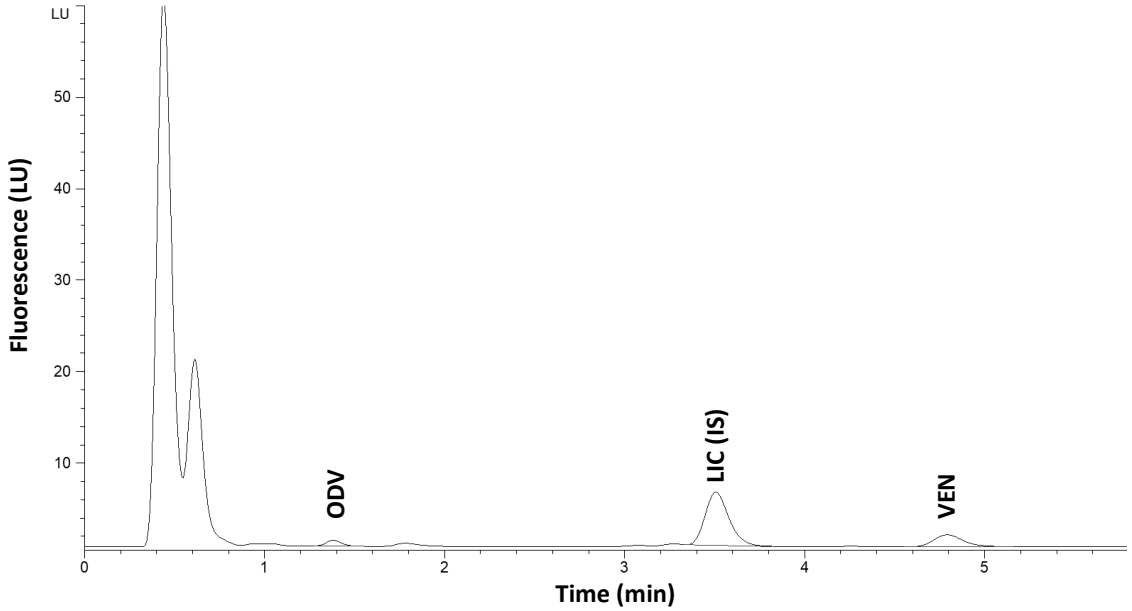


Figure II.2.4 Representative chromatogram of the analysis of real plasma samples obtained from patients under treatment with venlafaxine. Specifically, this chromatogram was generated by the analysis of the sample collected from patient ID5. LIC, Licarbazepine; ODV, *O*-desmethylvenlafaxine; VEN, Venlafaxine.

From the five concentrations determined, only the ODV concentration in the ID_2 and the VEN concentration in the ID_5 were in agreement with the established therapeutic window for each one of analytes (70-300 ng/mL for VEN and 200-500 ng/mL for ODV). In fact, with exception of the VEN concentration in the ID_2, the concentrations were below to the corresponding lower limit of the therapeutic range. However, it has also been recommended that the concentration of active portion (VEN plus ODV) should be within 100-400 ng/mL in order to optimize the binominal efficacy/toxicity (70). Thus, bearing this in mind, the daily doses of VEN could be increased for the ID_1, ID_3, ID_4 and ID_5 if an improved antidepressant response is required. Undoubtedly, all these findings confirm the validity and the applicability of this bioanalytical assay in the clinical practice as a tool for therapeutic drug monitoring of VEN, in order to guide the individualization of dosing regimens.

II.2.4 CONCLUSIONS

A new and reliable HPLC-FLD assay was herein reported to simultaneously quantify VEN and ODV in human plasma using, for the first time, a sample preparation procedure based on MEPS. The method presents several important bioanalytical advantages. Beyond its high cost-effectiveness, the plasma sample volume required is very low (100 µL), enabling the use of less invasive sampling collection procedures and performing additional analyses from the same sample when necessary. Moreover, the chromatographic analysis is quite fast and the sample preparation approach is also relatively simple and fast, which translates in high throughput analysis (about 30 minutes per analysis). All these aspects, associated with the less expensive technology required, make of this new bioanalytical HPLC assay an attractive and promising alternative for the routine therapeutic drug monitoring of VEN, as well as for other clinical pharmacokinetic and toxicological-based studies.

II.2.5 FUTURE PERSPECTIVE

Attending to the state of art of the antidepressant pharmacotherapy, we believe, by their noticeable advantages, that this new bioanalytical methodology will represent an important tool at the service of therapeutic drug monitoring in psychiatry, constituting a stimulus to the implementation of the pharmacokinetic monitoring in the clinic, guiding the therapeutic decisions/interventions. Furthermore, this highly throughput and cost-effective technique opens the door for more robust clinical pharmacokinetics and pharmacogenetics/pharmacokinetics based-studies.

Actually, the method herein described clearly reflects a paradigm shift in the field of bioanalytical sciences, particularly in what concerns to the application of new sample preparation methodologies in the clinical practice.

In the future, more than miniaturized and automated devices, it will potentially be possible to carry out quantitative determinations of drugs and other compounds by only one single device/step and through more convenient sampling systems, such as online or dried blood spot systems.

II.3 THERAPEUTIC DRUG MONITORING OF FLUOXETINE, NORFLUOXETINE AND PAROXETINE: A NEW TOOL BASED ON MICROEXTRACTION BY PACKED SORBENT COUPLED TO LIQUID CHROMATOGRAPHY

II.3 THERAPEUTIC DRUG MONITORING OF FLUOXETINE, NORFLUOXETINE AND PAROXETINE: A NEW TOOL BASED ON MICROEXTRACTION BY PACKED SORBENT COUPLED TO LIQUID CHROMATOGRAPHY

II.3.1 INTRODUCTION

FLU and PAR are antidepressant agents belonging to the class of SSRIs, which are currently the gold-standard treatment for depression (65,74,273). However, antidepressant drug therapy has shown a high variability in the individual's response, ranging from severe adverse effects to failure or delayed therapeutic response (147,159,160,240). Therefore, in recent years, efforts have been concentrated in establishing more effective and safer antidepressant regimens, considering the individual characteristics of patients.

At this level, therapeutic drug monitoring of antidepressants has been enunciated as a useful tool and, specifically, the monitoring of the plasma levels of FLU plus NFLU and PAR has demonstrated therapeutic and economic benefits (70,196,241,273,276,277). On this matter, it is important to underline that the therapeutic drug monitoring of FLU should take into account the concentrations of the drug itself (FLU), as well as of its main metabolite NFLU; in fact, NFLU is a pharmacologically active metabolite of FLU with comparable potency. On the other hand, PAR has no active metabolites and, in this case, the therapeutic drug monitoring recommendations are focused only on the concentration of the parent drug (70,276). Accordingly, therapeutic drug monitoring of FLU and PAR has been recommended mainly for dose titration, special populations and/or circumstances (e.g. children, elderly, pregnant or breast feeding patients, high potential for clinically relevant drug interactions and pharmacokinetically important co-morbidities) or problems solving, such as therapeutic failure and/or toxicity (70). Hence, the availability of reliable and cost-effective bioanalytical methods that allow the simultaneous quantification of FLU and NFLU, and the quantification of PAR, is a prerequisite for therapeutic drug monitoring interventions targeting these antidepressant drugs in the routine clinical practice (70,276,277).

Over the years, numerous liquid chromatography (LC) methods (247,248,280–285,250,271–273,276–279) have been reported for the quantitative analysis of FLU, NFLU

and PAR in human plasma and serum. Nevertheless, the majority of them are based on LLE (247,250,271,277,281,285) or SPE (248,277,282–285) procedures and many do not contemplate the simultaneous analysis of FLU and its active metabolite NFLU (272,277,278,280,283–285). However, the recent trends in bioanalysis involve the miniaturization, high-throughput performance, automation and even the on-line coupling of sample preparation and instrumental analysis. In this context, MEPS, a miniaturized version of the conventional SPE, has gained a considerable relevance (258,278,286–291).

Actually, MEPS has been successfully used in the determination of a wide variety of drugs and metabolites, including antidepressants (258,278,287,288). For instance, Chaves *et al.* described a MEPS/LC-UV method for the determination of sertraline, mirtazapine, fluoxetine, citalopram and paroxetine in human plasma (278); and, more recently, Alves *et al.* also proposed a MEPS/LC-DAD assay for the quantification of FLU, NFLU, clomipramine and desmethylclomipramine in human urine (287). Nonetheless, to the best of our knowledge, there is no method described for the simultaneous quantification of FLU and NFLU in human plasma based on the MEPS technology.

Thus, this work aimed to develop and fully validate the first MEPS/LC assay for the simultaneous analysis of FLU and NFLU, and also PAR in human plasma.

II.3.2 MATERIALS AND METHODS

II.3.2.1 CHEMICALS AND REAGENTS

Analytical standards of FLU, NFLU, PAR and VEN used as IS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol of LC gradient grade were obtained from Fisher Scientific (Leicestershire, UK). All other reagents were of analytical grade: sodium phosphate monobasic anhydrous (Acros Organics SA; Barcelona, Spain), di-potassium hydrogen phosphate anhydrous (Panreac Química SA; Barcelona, Spain), ammonium hydroxide (24.5 %) (J.T.Baker; Deventor, Holland), and formic acid (98-100 %) (Merck; Darmstadt, Germany). MEPS 250 µL syringe and MEPS BIN (barrel insert and needle) containing ~4 mg of solid-phase silica – C8 material (SGE Analytical Science, Australia) were purchased from ILC (Porto, Portugal). Ultra-pure water (LC grade, >18 MΩ.cm) was obtained from a Milli-Q water apparatus (Millipore; Milford, MA, USA).

II.3.2.3 PLASMA SAMPLES

Drug-free plasma from healthy blood donors was provided by Portuguese Blood Institute. Real plasma samples were obtained from adult outpatients with depression under therapy with FLU and PAR and followed at the Health Centre of Covilhã (Covilhã, Portugal). The clinical protocol for blood sample collection was approved by a Competent Ethics Committee and all samples were obtained in accordance with the principles of Helsinki Declaration and after the written informed consent to be given.

II.3.2.4 STOCK SOLUTIONS, CALIBRATION STANDARDS AND QUALITY CONTROL SAMPLES

Stock and intermediate solutions were appropriately used to prepare six combined spiking solutions in ultra-pure water/methanol (75:25, v/v) at final concentrations of 0.5, 1, 3, 6.25, 12.5 and 18.75 µg/mL for FLU and NFLU, and 0.125, 0.25, 1.25, 5, 12.5 and 18.75 µg/mL for PAR. These combined solutions were used to spike blank human plasma in order to prepare the calibration standards at six different concentration levels for FLU and NFLU (20, 40, 120, 250, 500 and 750 ng/mL) and for PAR (5, 10, 50, 200, 500 and 750 ng/mL). An IS working solution was daily prepared also in water–methanol (75:25, v/v) at 2.5 µg/mL. QC samples at the lower limit of quantification (QC_{LLOQ}) and at low (QC_1), middle (QC_2) and high (QC_3) concentrations representative of the calibration ranges were also independently prepared in the same biological matrix. To evaluate the dilution integrity (1:10) another QC sample was prepared (QC_{dil}).

II.3.2.5 INSTRUMENTATION AND CHROMATOGRAPHIC CONDITIONS

The chromatographic analysis was carried out on an UHPLC system (Agilent 1290 Infinity Binary LC system) equipped with a FLD detector (Agilent 1260 Infinity; G1321B). Chromatographic separation of FLU, NFLU, PAR and IS was accomplished in less than 13 min on a reversed-phase LiChroCART® Purospher Star-C18 column (55 × 4 mm; 3 µm particle size from Merck KGaA, Darmstadt, Germany), employing isocratic elution at a flow-rate of 1 mL/min and a column temperature of 45 °C. The mobile phase was

composed by an aqueous solution of 25 mM sodium phosphate monobasic anhydrous and 7.5 mM di-potassium hydrogen phosphate anhydrous (pH 3.0)/acetonitrile/methanol (70:23:7, v/v/v). The injection volume was 40 µL and the excitation/emission wavelengths were set at 240/312 nm for the detection of FLU, NFLU and IS and at 295/350 nm for the detection of PAR.

II.3.2.6 SAMPLE PREPARATION AND MEPS EXTRACTION

To each aliquot of human plasma sample (500 µL), spiked with 20 µL of the IS working solution, was added 1.5 mL of acetonitrile in order to precipitate the plasma proteins. This mixture was vortex-mixed for 60 s and centrifuged at 13000 rpm for 3 min. The resulting supernatant was evaporated to dryness under a gentle nitrogen stream at 50 ºC; then, the dried residue was reconstituted with 500 µL of 50 mM sodium phosphate monobasic anhydrous aqueous solution (pH 4.0).

Afterwards, the reconstituted sample extract was subjected to the MEPS procedure, which was manually carried out (off-line) using a C8 MEPS cartridge. Before the first extraction, the MEPS cartridge was activated with 3 × 200 µL of methanol and conditioned with 2 × 200 µL of ultra-pure water. After that, all the volume of the reconstituted extract was drawn through the MEPS sorbent and ejected for three times (cycles). The sorbent was then washed with 2 × 200 µL of 5% ammonium hydroxide aqueous solution and the analytes were eluted with 5 × 200 µL of methanol with 1% of formic acid. Finally, the eluate was evaporated to dryness under a gentle stream of nitrogen at 50 ºC, redissolved in 100 µL of mobile phase and injected into the chromatographic system. After each extraction, the MEPS sorbent was sequentially washed/reconditioned with 10 × 200 µL of methanol and 2 × 200 µL of ultra-pure water.

II.3.2.7 METHOD VALIDATION

The method validation was performed in agreement with the international recommendations for bioanalytical method validation issued by the Food and Drug Administration and the European Medicines Agency (259,260). The studied parameters

were selectivity, linearity, LLOQs, LODs, precision, accuracy, sample dilution integrity, recovery and stability.

Selectivity was evaluated by analysing the presence of chromatographic interferences from human plasma endogenous compounds at the retention times of FLU, NFLU, PAR and IS; for that, blank human plasma samples obtained from six different individuals were analysed and compared with spiked samples. On the other hand, the selectivity of the assay was also tested against the chromatographic interference from other potentially co-administered drugs such as antidepressants (amitriptyline, citalopram, clomipramine, duloxetine, dosulepin, escitalopram, fluvoxamine, maprotiline, mirtazapine, nortriptyline, sertraline and trazodone), anxiolytics, sedatives and hypnotics (alprazolam, diazepam and zolpidem), antiepileptics (carbamazepine, lamotrigine, primidone and valproic acid), antibiotics (ciprofloxacin, clinafloxacin, levofloxacin, moxifloxacin, norfloxacin and pefloxacin), antihypertensives (furosemide, metoprolol and verapamil), analgesics, antipyretics and anti-inflammatory drugs (paracetamol, acetylsalicylic acid, ketoprofen and piroxicam), and other drugs as risperidone, caffeine, cimetidine, digoxin, hydrocortisone, nicotine, quinidine, tamoxifen, theophylline and warfarin; in this case, the chromatographic selectivity was evaluated by direct chromatographic analysis of standard solutions of these drugs at 10 µg/mL.

The linearity of the analytical method was evaluated through the construction of three calibration curves on three distinct days (n = 3), using six calibration standards in the ranges of 20-750 ng/mL for FLU and NFLU, and 5-750 ng/mL for PAR. The calibration curves were obtained by plotting analytes/IS peak area ratios *versus* the corresponding nominal plasma concentrations, considering a weighted linear regression analysis (261).

The LLOQs, defined as the lowest concentration of the calibration curve that can be measured with acceptable precision and accuracy, were assessed by the CV values equal or lower than 20% and the deviation from nominal concentration (*bias)* values within ±20%. The LODs were determined by a signal-to-noise approach and were established as the concentrations that yields a signal/noise ratio of 3/1.

Inter-day precision and accuracy were assessed by analysing each QC sample (QC_{LLOQ}, QC_1, QC_2, QC_3 and QC_{dil}) in three distinct days (n = 3), whereas the intra-day precision and accuracy were assessed by analysing five sets of QC samples in a single day (n = 5). In the analysis of the dilution integrity, a ten-fold (1/10) dilution step with blank

human plasma was performed before extraction of each QC_{dil} sample. In this scope, the acceptance criterion for precision was a CV value ≤ 15% (or 20% in the QC_{LLOQ}) and for accuracy was a *bias* value within ±15% (or ±20% in the QC_{LLOQ}).

The recovery of FLU, NFLU and PAR from human plasma samples was evaluated through the analysis of five replicate samples (*n* = 5) at each one of the QC_1, QC_2 and QC_3 concentration levels. Absolute recovery was calculated by comparing the peak area of the analytes from extracted QC samples with the corresponding peak area obtained from the direct injection of non-extracted solutions at the same nominal concentrations (*n* = 5).

Stability of FLU, NFLU and PAR in human plasma was assessed by comparing the data of QC_1 and QC_3 samples analysed in replicate (*n* = 5) before (reference samples) and after being exposed to the conditions for stability assessment (stability samples). Spiked QC plasma samples were exposed at room temperature for 4 h, at 4 ºC for 24 h, at −20 ºC for 30 days and at three freeze-thaw cycles (i.e., samples were stored at −20 ºC during 24 h, thawed unassisted at room temperature and then refrozen under the same conditions until completion of the three cycles). Additionally, post-preparative stability was also studied; processed samples were analysed after 12 h at room temperature in the autosampler. A stability/reference samples ratio within 85–115% was accepted as the stability criterion.

II.3.3 RESULTS AND DISCUSSION

II.3.3.1 OPTIMIZATION OF THE MEPS PROTOCOL

The sample preparation procedure herein proposed was developed and optimized considering the MEPS conditions described by Chaves *et al.* for analysis of antidepressants (sertraline, mirtazapine, fluoxetine, citalopram and paroxetine) in human plasma (278). Specifically, the MEPS procedure was adapted to include the pharmacologically active FLU metabolite (NFLU) and to improve the efficiency and cost-utility of the method.

Nowadays, it is clear that MEPS procedures require the use of adequate sample pre-treatment strategies, which depend on the target biological matrix, in order to avoid the rapid clogging of MEPS cartridges (258,264,268,286). Chaves *et al.* proposed a dilution step of the plasma sample with 50 mM phosphate buffer solution (pH 4.0; 1:1, v/v) before

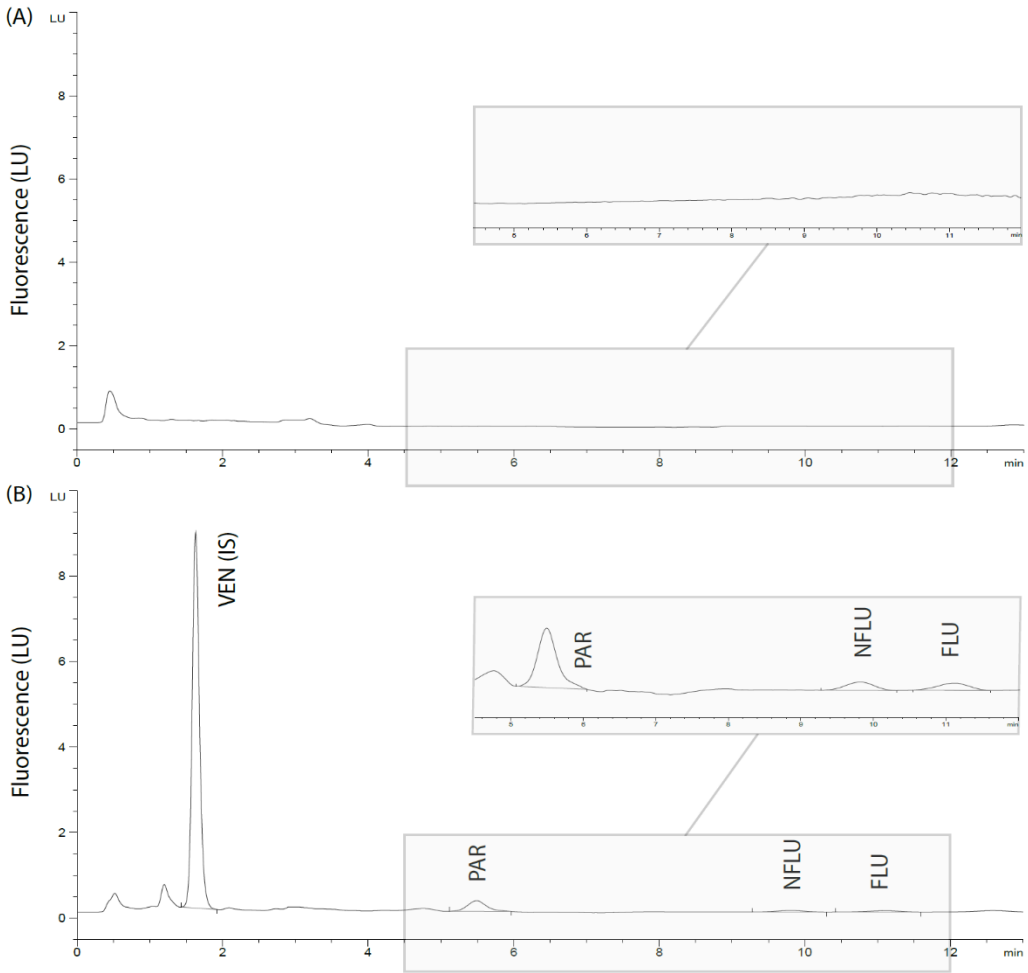
sample loading (278). However, when this strategy was applied in our preliminary assays, the MEPS cartridge clogged after less than 10 extractions. Actually, different sample pre-treatment approaches have been used before MEPS procedures but, consistent with our experience, the sample deproteinization is demanded when the matrix is human plasma (258,264,268,286). Thus, we tested several protein precipitating agents including trichloroacetic acid (20%), saturated ammonium sulphate, methanol and acetonitrile (1:3, v/v); among them, only acetonitrile ensured an effective clean-up of the sample with acceptable recoveries. This approach enabled to obtain cleaner chromatograms and to extend the lifetime of the MEPS cartridge for about 80 extractions.

Specifically considering the MEPS protocol, the sorbent activation/conditioning was defined *a priori* as 2 × 200 µL of methanol and 2 × 200 µL of water, because these conditions are the most frequently used in this step (258). Although Chaves et al. have used M1 (C8+SCX) MEPS cartridges, the comparative performance of M1, C8 and C18 MEPS cartridges was preliminarily assessed, and the obtained results showed an improved extraction efficiency with C8 MEPS cartridges for all compounds. The number of loading draw-eject cycles was also evaluated considering 1, 3 and 5 cycles, but the results obtained showed to be in line with those observed by Chaves *et al.*; all the compounds (FLU, NFLU, PAR and IS) displayed the maximum recovery using three draw-eject cycles. The elution and washing conditions were also modified to enhance the recovery and selectivity of the method. On the one hand, the use of 1 mL of methanol as elution strategy significantly increased the absolute recovery of all the analytes, in particular of NFLU; on the other hand, the basification and acidification of the washing and elution solutions, respectively, was needed to remove interferences at the retention times of FLU and IS. As a result, the use of 200 µL of 5% ammonium hydroxide aqueous solution for the washing step and 5 x 200 µL of methanol with 1% of formic acid for the elution step were found to be the best conditions. Lastly, the carryover was also investigated and, to avoid memory effects, it is needed to clean the cartridge with 10 x 200 µL of methanol and 2 × 200 µL of water after each extraction.

II.3.3.2 VALIDATION

II.3.3.2.1 SELECTIVITY

No endogenous neither exogenous interferences (tested drugs) were found at the retention times of FLU, NFLU, PAR and IS. Considering the set of tested drugs only metoprolol (0.99 min), verapamil (7.91 min), moxifloxacin (1.05 min), clinafloxacin (0.45 min), acetylsalicylic acid (1.33 min), quinidine (0.96 min), norfloxacin (0.61 min), escitalopram (3.52 min) and zolpidem (1.41 min) were detected. The chromatograms of blank and spiked human plasma samples are shown in Figure II.3.1.



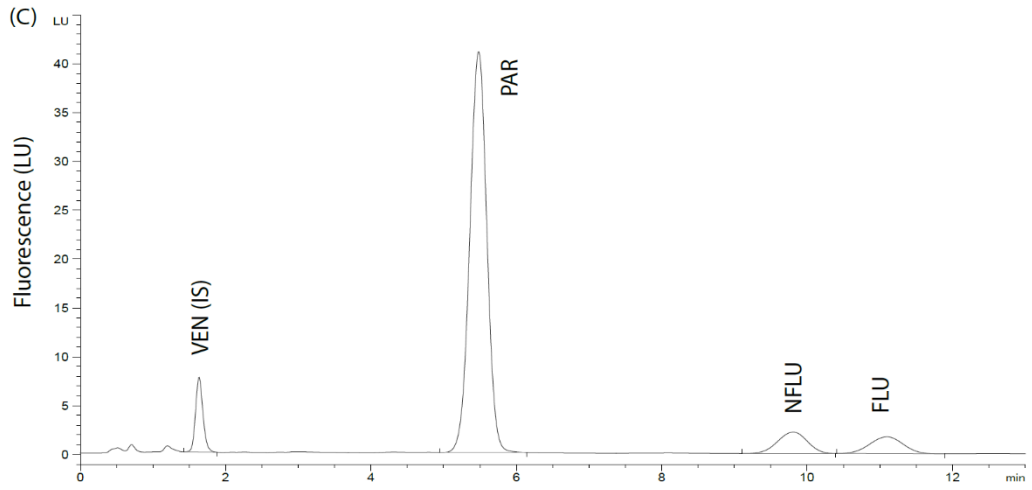


Figure II.3.1 Representative chromatograms of the analysis of human plasma samples: (A) blank plasma; (B) plasma spiked with internal standard [IS; venlafaxine (VEN)] and the analytes [paroxetine (PAR), norfluoxetine (NFLU) and fluoxetine (FLU)] at concentrations of the lower limit of quantification (5 ng/mL for PAR and 20 ng/mL for NFLU and FLU), and (C) at concentrations of the upper limit of calibration range (750 ng/mL for all the analytes).

II.3.3.2.2 LINEARITY, LLOQ AND LOD

The method was linear ($r^2 \geq 0.9919$) over the concentration range of 20-750 ng/mL for FLU and NFLU and 5-750 ng/mL for PAR. Heteroscedasticity was concluded for all the analytes and, hence, weighted linear regression analysis was applied; among the several weighting factors evaluated for each analyte ($1/\sqrt{x}$, $1/x$, $1/x^2$, $1/\sqrt{y}$, $1/y$ and $1/y^2$), $1/x^2$ was the best-fitting factor for all of them. The weighted regression equations (n = 3) of the calibration curves using $1/x^2$ as weighting factor were $y = 0.00109\,x - 0.00734$ ($r^2 = 0.9946$) for FLU; $y = 0.00121\,x - 0.00854$ ($r^2 = 0.9919$) for NFLU and $y = 0.01271\,x + 0.00360$ ($r^2 = 0.9932$) for PAR, where y represents the analyte/IS peak area ratio and x represents the plasma concentration. The LLOQ was set at 20 ng/mL for FLU and NFLU and 5 ng/mL for PAR. The LOD was established at 5 ng/mL for FLU and NFLU and 1 ng/mL for PAR.

In order to critically evaluate the performance of the MEPS/LC-FLD assay herein reported, in the Table II.3.1 is provided a comparative view of several determinant bioanalytical aspects between the current method and LC assays coupled to conventional (low-cost) detection systems (LC-UV, LC-DAD and LC-FLD) previously developed for the bioanalysis of FLU, NFLU and PAR in human plasma, serum and urine. Overall, bearing in

mind that we use a miniaturized extraction procedure, the calibration ranges and LLOQs achieved with our method are very interesting comparatively to those obtained by homologues methodologies (Table II.3.1) (247,248,280–285,287,292,293,250,271–273,276–279). When compared with other MEPS/LC assays already developed for these analytes, our LLOQ values are the same or even lower; specifically, Chaves *et al.* obtained a LLOQ of 20 ng/mL for FLU and PAR in human plasma (278) and Alves *et al.* reported a LLOQ value of 100 ng/mL for FLU and NFLU in human urine (287). As shown in Table II.3.1, it is also worthy to underline that some of the currently available methods based on microextraction techniques achieved lower LLOQ values (279,280,292); however, for the majority of them, a higher sample volume is required for analysis (279,280). At this point, it should be acknowledged, that apart from these methods, there are others available in literature that allow a faster analysis using smaller sample volume, but such methods require more expensive instrumentation [e.g., LC-tandem mass spectrometry (MS/MS)] (285). Lastly, the current method seems to be a useful tool, not only for pharmacokinetics or toxicological purposes (70,279), but also to study in depth pharmacogenetics/pharmacokinetics relationships, particularly for FLU, because this assay enables the simultaneous determination of the parent drug and its active metabolite NFLU; indeed, the conversion of FLU to NFLU is mediated by highly polymorphic cytochrome P450 isoenzymes, mainly the CYP2D6 (98).

Table II.3.1 Comparison of determinant bioanalytical aspects between the current method and other liquid chromatography methods (LC-UV, LC-DAD and LC-FLD) previously developed for the bioanalysis of fluoxetine (FLU), norfluoxetine (NFLU) and paroxetine (PAR) in human plasma, serum and urine.

Extraction	Analytical system	Analytes	Sample (Volume)	Calibration range	Recovery	Reference
MEPS	**LC-FLD**	**FLU, NFLU**	**Plasma (0.5 mL)**	**20-750 ng/mL**	**58.7-77.3%**	**Current method**
		PAR		**5-750 ng/mL**		
	LC-UV	FLU, PAR	Plasma (0.4 mL)	20-1000 ng/mL	NA	(278)*
	LC- DAD	FLU, NFLU	Urine (0.5 mL)	100-5000 ng/mL	90.4-98.8%	(287)*
SPME	LC-UV	FLU	Plasma (0.5 mL)	1-500000 ng/mL	79.0-94.0%	(292)*
			Urine (0.5 mL)			
DPX	LC-FLD	FLU	Plasma (0.2 mL)	10-1000 ng/mL	74.87-74.91%	(293)
		NFLU		80-1000 ng/mL		
LPME	LC-FLD	FLU, NFLU	Plasma (1.0 mL)	5-500 ng/mL	52.0-76.9%	(279)
	LC-FLD	FLU, PAR	Plasma (1.0 mL)	5-500 ng/mL	64.9-71.3%	(280)*
USAEME	LC-UV	FLU	Plasma (1.25 mL)	25-1000 ng/mL	47.7-49.5%	(272)*
			Urine (1.25 mL)			
SPE	LC-UV	FLU, NFLU	Serum (1.0 mL)	10-500 ng/mL	95.2-107.0%	(248)*
		PAR		5-500 ng/mL		
	LC-UV	FLU, PAR	Plasma (0.05 mL)	1200-20000 ng/mL	83.0-109.6%	(284)*
			Urine (0.1 mL)	1800-20000 ng/mL		

Table II.3.1 Comparison of determinant bioanalytical aspects between the current method and other liquid chromatography methods (LC-UV, LC-DAD and LC-FLD) previously developed for the bioanalysis of fluoxetine (FLU), norfluoxetine (NFLU) and paroxetine (PAR) in human plasma, serum and urine.

Extraction	Analytical system	Analytes	Sample (Volume)	Calibration range	Recovery	Reference
LLE	LC-DAD	FLU	Serum (1.0 mL)	15-1000 ng/mL	83.7-96.3%	(271)*
		NFLU		25-1000 ng/mL		
		PAR		20-500 ng/mL		
	LC-DAD	FLU, NFLU, PAR	Plasma (NA)	25-1000 ng/mL	74.0-109.0%	(247)*
	LC-DAD	FLU, NFLU, PAR	Plasma (NA)	25-500 ng/mL	NA	(250)*

DAD, Diode array detection; DPX, Disposable pipette extraction; FLD, Fluorescence detection; LC, Liquid chromatography; LLE, Liquid-liquid extraction; LPME, Liquid-phase microextraction; MEPS, Microextraction by packed sorbent; NA, not available; SPE, Solid-phase extraction; SPME, Solid-phase microextraction; USAEME, Ultrasound-assisted emulsification microextraction; UV, Ultraviolet detection. *This method determines other antidepressant(s).

II.3.3.2.3 PRECISION AND ACCURACY

The overall intra- and inter-day precision (CV values) did not exceed 13.6 % and the corresponding accuracy (*bias* values) ranged from 0.02 to 16.7 % (Table II.3.2). Thus, this assay is accurate, precise and reproducible, including when the dilution of a plasma sample is required.

Table II.3.2 Inter- and intra-day precision and accuracy for the quantification of fluoxetine (FLU), norfluoxetine (NFLU) and paroxetine (PAR) in human plasma samples at the concentrations of the lower limit of quantification (QC_{LLOQ}) and at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges and following the sample dilution by a 10-fold factor (QC_{dil}).

Analyte	Nominal concentration (ng/mL)	Experimental concentration (mean ± SD, ng/mL)	Precision (% CV)	Accuracy (% *bias*)
Inter-day (n = 3)				
FLU	20 (QC_{LLOQ})	23.2 ± 1.1	6.4	16.0
	60	63.9 ± 4.4	7.8	6.4
	375	357.4 ± 17.5	5.0	-4.7
	675	626.6 ± 55.2	8.9	-7.2
	2000 (QC_{dil})	1873.8 ± 142.1	7.9	-6.3
NFLU	20 (QC_{LLOQ})	21.5 ± 1.4	9.6	7.5
	60	63.1 ± 2.2	3.9	5.1
	375	366.7 ± 16.4	4.6	-2.2
	675	676.6 ± 27.1	4.0	0.2
	2000 (QC_{dil})	1957.9 ± 71.2	3.8	-2.1
PAR	5 (QC_{LLOQ})	5.4 ± 0.1	4.9	6.9
	15	15.3 ± 0.8	2.6	1.9
	375	364.0 ± 0.3	0.1	-2.9
	675	686.4 ± 33.7	4.9	1.7

Table II.3.2 Inter- and intra-day precision and accuracy for the quantification of fluoxetine (FLU), norfluoxetine (NFLU) and paroxetine (PAR) in human plasma samples at the concentrations of the lower limit of quantification (QC_{LLOQ}) and at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges and following the sample dilution by a 10-fold factor (QC_{dil}).

Analyte	Nominal concentration (ng/mL)	Experimental concentration (mean ± SD, ng/mL)	Precision (% CV)	Accuracy (% *bias*)
Intraday (n = 5)				
FLU	20 (QC_{LLOQ})	23.3 ± 1.6	11.2	16.7
	60	63.4 ± 7.4	13.6	5.7
	375	359.2 ± 15.9	4.5	-4.2
	675	669.6 ± 40.4	6.1	-0.8
	2000 (QC_{dil})	1893.4 ± 74.5	4.1	-5.3
NFLU	20 (QC_{LLOQ})	22.1 ± 1.7	12.4	10.7
	60	64.4 ± 6.2	11.2	7.3
	375	376.5 ± 21.5	5.9	0.4
	675	682.2 ± 44.7	6.6	1.1
	2000 (QC_{dil})	1980.7 ± 82.4	4.4	-1.0
PAR	5 (QC_{LLOQ})	5.7 ± 0.5	8.6	13.0
	15	15.1 ± 1.8	12.1	0.5
	375	375.1 ± 23.8	6.4	0.02
	675	721.00 ± 58.6	8.1	6.8
	2000 (QC_{dil})	1838.1 ± 73.9	4.0	-8.1

CV, coefficient of variation; SD, standard deviation.

II.3.3.2.4 ABSOLUTE RECOVERY

The mean recoveries ranged from 58.9 to 65.2% for FLU, 58.7 to 66.9% for NFLU and 70.4 to 77.3% for PAR with acceptable CV values (Table II.3.3). In turn, the mean recovery of the IS was 70.9% with a CV of 11.1%.

Table II.3.3 Absolute recovery (%) of fluoxetine (FLU), norfluoxetine (NFLU) and paroxetine (PAR) from human plasma samples at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges (n = 5).

Analyte	Nominal concentration (ng/mL)	Recovery (%) (mean ± SD)	CV (%)
FLU	60	61.9 ± 7.2	11.6
	375	65.2 ± 6.2	9.6
	675	58.9 ± 2.3	3.8
NFLU	60	61.2 ± 5.3	8.6
	375	66.9 ± 6.9	10.3
	675	58.7 ± 2.6	4.4
PAR	15	76.9 ± 3.6	4.8
	375	77.3 ± 7.6	9.8
	675	70.4 ± 4.9	7.0

CV, coefficient of variation; SD, standard deviation.

On this matter, the extraction efficiency of this assay is in line with the previous results achieved with MEPS (258). However, it is important to recognise that the sample pre-treatment step, which was considered to improve the lifetime of the MEPS cartridge, may also contribute to some loss of the analytes. Therefore, it would be plausible that similar assays could report higher extraction efficiencies (Table II.3.1). Once again, Chaves *et al.* did not report the absolute recovery achieved in human plasma (278), and a direct comparison with the MEPS/LC assay developed by Alves *et al.* can be ambiguous, because urine is a less complex matrix wherein a sample dilution strategy is often enough before the MEPS procedure (287).

II.3.3.2.5 STABILITY

No significant degradation occurred for FLU, NFLU and PAR in human plasma at room temperature for 4 h, at 4 ºC for 24 h, after three freeze-thaw cycles at -20 ºC and at

-20 ºC for 30 days, neither in processed plasma samples at room temperature during 12 h (Table II.3.4).

Table II.3.4 Stability (values in percentage) of fluoxetine (FLU), norfluoxetine (NFLU) and paroxetine (PAR) under the different conditions tested (*n* = 5).

Analytes	**FLU**		**NFLU**		**PAR**	
Nominal concentration (ng/mL)	**60**	**675**	**60**	**675**	**15**	**675**
Unprocessed plasma						
Room temperature (4 h)	95.6	95.0	85.5	95.6	87.7	99.6
4 ºC (24 h)	110.9	93.6	111.7	100.1	111.0	106.4
Freeze/thaw (3 cycles; −20 ºC)	111.5	93.4	111.6	92.2	106.2	101.9
−20 ºC (30 days)	99.2	96.0	99.1	93.6	104.9	98.5
Processed plasma						
Room temperature (12 h)	110.2	103.9	113.9	111.2	101.9	104.8

II.3.3.2.6 CLINICAL APPLICATION TO AUTHENTIC PLASMA SAMPLES

The MEPS/LC-FLD assay herein described was successfully used to quantify FLU, NFLU and PAR in plasma samples taken from depressed patients. The prescribed dosage regimen for each individual (ID), the time after the last dose, the co-medication and the respective drug concentrations determined are summarized in Table II.3.5.

Table II.3.5 Plasma concentrations of fluoxetine (FLU) and its active metabolite norfluoxetine (NFLU), and paroxetine (PAR) determined in authentic human plasma samples obtained from depressive outpatients taking FLU or PAR *per os*. Data on the corresponding prescribed regimen, time after the last dose and co-medication are also presented.

Patients	Drug	Regimen (dose/day)	Time after the last dose (hh:mm)	Concentration (ng/mL)		Co-medication
				FLU	**NFLU**	
ID_1	FLU	20 mg	07:20	107.1	146.6	Atorvastatin, calcium carbonate, cholecalciferol, fluticasone propionate, folic acid, lisinopril, methotrexate, pantoprazole, prednisolone, salmeterol, sulfasalazine
ID_2	FLU	40 mg	05:15	415.7	553.3	Celecoxib, cyclobenzaprine, valproic acid, trazodone
ID_3	FLU	20 mg	22:55	100.0	149.5	Amitriptyline, betahistine, digoxine, enalapril, idebenone, lorazepam, pantoprazole, simvastatin, ticlopidine, trimetazidine
				PAR		
ID_4	PAR	40 mg	13:40	68.6		Alprazolam, enalapril, esomeprazole, simvastatin
ID_5	PAR	20 mg	06:45	191.1		Allopurinol, atorvastatin, candesartan, diazepam, omeprazole, paracetamol, warfarin

No interferences were verified at the retention time of the analytes (FLU, NFLU, PAR and IS) in any of the real plasma samples analysed, including from the co-prescribed drugs (Table II.3.5). Indeed, the chromatographic profile of the analytes and IS in real plasma samples is similar to that obtained after the analysis of spiked blank human plasma samples. Representative chromatograms of authentic plasma samples from patients treated with FLU (ID_2) and PAR (ID_5) are depicted in the Figure II.3.2.

Analysing the results, the plasma concentrations determined for the ID_1, ID_3, and ID_4 are within the respective therapeutic ranges that have been proposed for each one of the analytes (50-500 ng/mL for FLU and NFLU and 30-120 ng/mL for PAR) (70,279).

Moreover, in order to improve the clinical outcomes, the FLU active portion (FLU plus NFLU) should be maintained within 120-500 ng/mL (70). Also, at this level, the concentrations measured for these two individuals under treatment with FLU (ID_1 and ID_3) are in accordance with the recommended therapeutic concentration range. However, if an improved antidepressant response is required, the daily doses of FLU and PAR could be carefully increased in these individuals, given the considerable differences between the measured concentrations and the corresponding upper limit of the therapeutic window/recommended range for the active moiety (FLU plus NFLU). On the other hand, the results warn for an augmented risk of adverse effects in the ID_2 and ID_5. Such results should be interpreted taking into account the clinical outcomes (efficacy *versus* adverse effects) and, if required, the dose of the antidepressant drugs can be decreased. In summary, these findings emphasize the need of monitoring the plasma concentrations of FLU, NFLU and PAR in the routine clinical practice, in order to achieve better clinical outcomes.

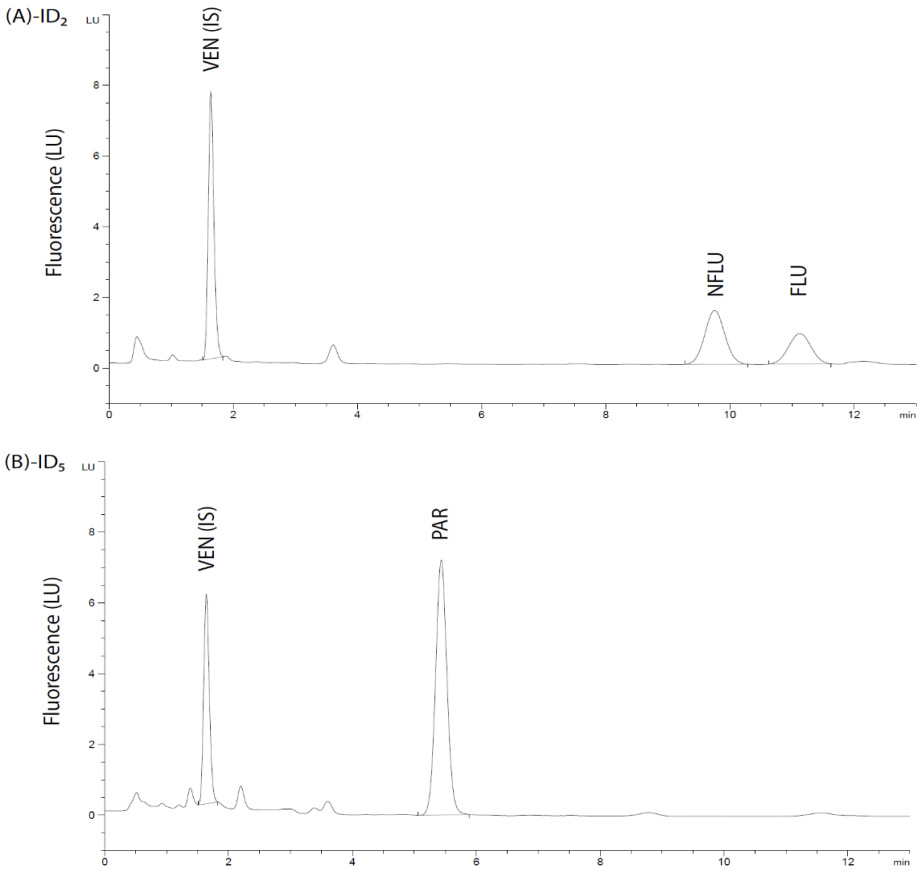


Figure II.3.2 Representative chromatograms of the analysis of authentic plasma samples obtained from: (A) ID_2, patient under treatment with fluoxetine (FLU) and (B) ID_5, patient under treatment with paroxetine (PAR).

II.3.4 CONCLUSION

A new and cost-effective MEPS/LC-FLD method was successfully developed and fully validated for the simultaneous quantification of FLU, NFLU and PAR in human plasma. This bioanalytical approach ensures simple, robust, low-cost, and environmentally friendly analysis, requiring a relatively small sample volume (500 µL) and minimum solvent consumption. Moreover, it allows the simultaneous quantification of FLU and its active metabolite NFLU, which is essential from a therapeutic drug monitoring point of view, and takes advantage from the MEPS technology, increasing the MEPS cartridge lifetime and decreasing the cost per analysis. On the other hand, this assay may be easily implemented in the majority of clinical laboratories, given the nature of the extraction conditions and analytical instrumentation involved. Thus, the new method herein proposed constitutes a good option for therapeutic drug monitoring of FLU and PAR and to support other clinical pharmacokinetic- and toxicological-based studies.

CHAPTER III

THE *GnG-PK/PD-AD* CLINICAL STUDY

III.1 GENERAL CONSIDERATIONS

III.1 GENERAL CONSIDERATIONS

After the bioanalytical development phase, the present doctoral work progressed for a clinical phase. This involved the planning, implementation and development of a real-world clinical study focused on the investigation of the impact of genetic and non-genetic factors on the pharmacokinetics and pharmacodynamics of widely prescribed antidepressant drugs: FLU, PAR and VEN, the so called *GnG-PK/PD-AD* clinical study.

The *GnG-PK/PD-AD* study was designed to fulfil two primary objectives. In a first phase, the study aimed to provide a real-world clinical characterization of Portuguese depressive patients treated with FLU, PAR and VEN, specifically in terms of pharmacokinetics and pharmacodynamics and clinical outcomes and the presence of clinically relevant genetic and non-genetic individual factors. This first objective corresponded to the exploratory analysis of the study data and it was planned to identify clinically relevant factors of inter-individual variability in the drug outcomes. In fact, the study of the influence of specific clinical variables on the drug outcomes is only justifiable if their variability is high and of potential clinical relevance. Thus, the characterisation of the inter-individual variability in the pharmacokinetics and pharmacodynamics (clinical outcomes) of FLU, PAR and VEN, as well as the characterisation of the inter-individual genetic and non-genetic variability, was considered of utmost importance to support and guide the subsequent analyses. Afterwards, the objective was to investigate the impact of the relevant individual genetic and non-genetic factors on the pharmacokinetics (plasma concentrations) and pharmacodynamics (clinical outcomes) of the antidepressant drugs in study, aiming at identifying potential therapeutic biomarkers.

Overall, the *GnG-PK/PD-AD* study was a multicentre, cross-sectional, real-world, clinical study carried out in collaboration with eight health units from the Center Region of Portugal and based on an Iberian partnership between the Laboratory of Pharmacology of the Faculty of Pharmacy of the University of Coimbra, Centre for Neuroscience and Cell Biology of the University of Coimbra (CNC), Health Sciences Research Centre of the University of Beira Interior (CICS-UBI) and the Clinical Research Centre of the Extremadura University Hospital and Medical School (CICAB). Figure III.1.1 schematically resumes the *GnG-PK/PD-AD* study.

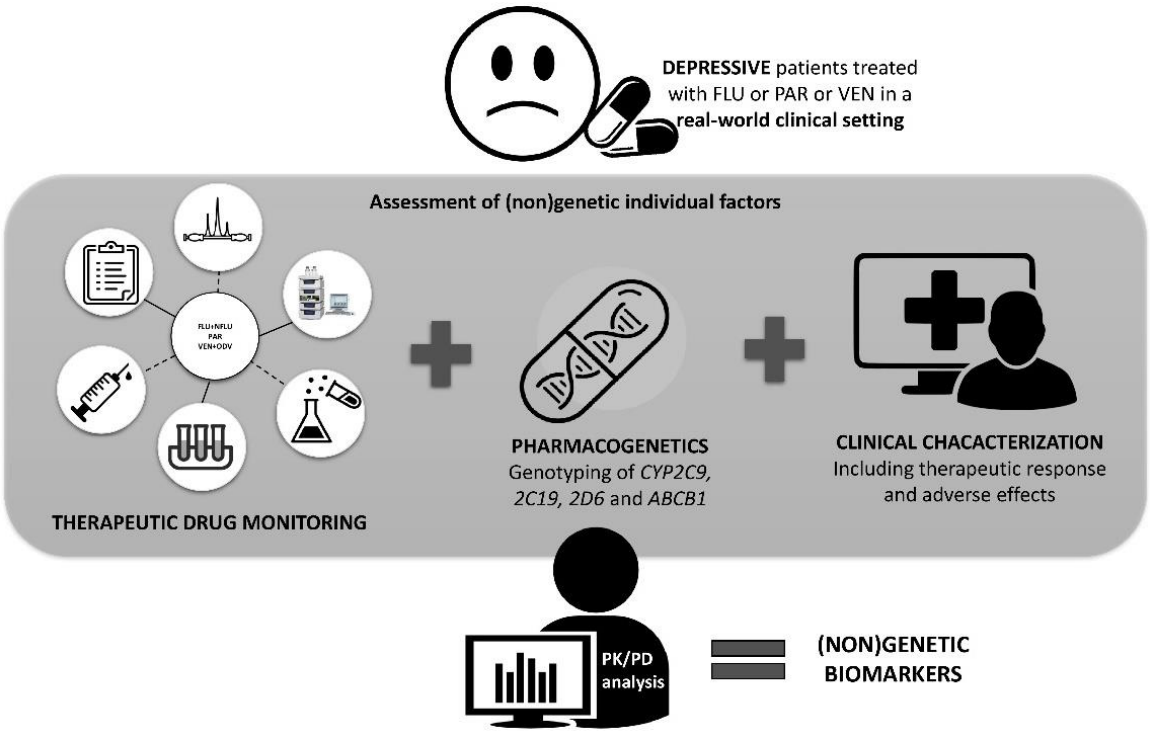


Figure III.1.1 Schematic representation of the *GnG-PK/PD-AD* study. ABCB1, P-glycoprotein gene; CYP, cytochrome P450; FLU, Fluoxetine; NFLU, norfluoxetine, ODV, *O*-desmethylvenlafaxine; PAR, paroxetine; VEN, venlafaxine.

Briefly, adult outpatients clinically diagnosed with depression and treated with FLU or PAR or VEN were recruited from the naturalistic clinical setting of the following health units of the Centre Region of Portugal: *Family Health Unit Condestável* (Batalha, Leiria), *Family Health Unit Ribeirinha* (Guarda, Portugal), *Family Health Unit Cruz de Celas* (Cruz de Celas, Coimbra), *Health Centre Covilhã* (Covilhã, Castelo Branco), *Health Centre Fundão* (Fundão, Castelo Branco), *Family Health Unit Topázio* (Eiras, Coimbra), *Coimbra Hospital and University Centre, Department of Psychiatry* (Coimbra), *Cova da Beira Hospital Centre* (Covilhã). The study involved more than 70 clinical collaborators, amongst medical, nursing and administrative staff. Eligible patients were invited to participate in the study by its physician during the routine clinical practice. Those who accepted to participate were subject to an appropriated informed consent process and were enrolled in the study. The study protocol involved just one visit in a single time-point (cross-sectional study). Patients were clinically and therapeutically characterized and then submitted to therapeutic drug monitoring (measurement of plasma drug concentration) of

drug/metabolite (FLU + NFLU or PAR or VEN + ODV) and genotyping of the *ABCB1, CYP2C9, CYP2C19* and *CYP2D6* genes, using the previously validated bioanalytical (Chapter II) and genotyping methods (173,183,294,295). Relevant individual data were gathered, and antidepressant clinical outcomes, including remission and adverse effects were assessed by means of the *17-item Hamilton Depression Rating Scale* (HAMD) and the *Antidepressant Side-Effect Checklist* (ASEC), respectively. The clinical data generated by the study were integrated and investigated by comprehensive pharmacokinetics/pharmacodynamics analyses, using advanced statistical tools. The drug bioanalysis phase of the study was carried out in the Analytical Facilities of the CICS-UBI. Genotyping analyses were performed in the Clinical Research Centre of the Extremadura University Hospital and Medical School (CICAB).

At this point, it should be highlighted that the study and all protocols were approved by the competent local Ethics Committees and by the CNPD – *Comissão Nacional de Protecção de Dados* – that is the Portuguese Data Protection Authority. The study was performed according to the principles of Good Clinical Practices and the Declaration of Helsinki and its subsequent revisions, the Council of Europe Convention on Human Rights and Biomedicine, the UNESCO Universal Declaration on the Human Genome and Human Rights and law in force for that purpose in Portugal and European Union, particularly the national law of data protection nº 67/98. All participants were extensively informed about the aspects of the study and were subjected to an appropriate informed consent process duly approved by the competent entities.

The present chapter will present the *GnG-PK/PD-AD* study and its results, which are included in the following original works:

- Magalhães P, Alves G, Fortuna A, Llerena A, Falcão A and Clinical collaborators of the GnG-PK/PD-AD study. Real-world clinical characterization of subjects with depression treated with antidepressant drugs focused on (non-)genetic factors, pharmacokinetics and clinical outcomes: GnG-PK/PD-AD study. *Exp Clin Psychopharmacol.* 2019. (ahead of print).
- Magalhães P, Alves G, Fortuna A, Llerena A, Falcão A and Clinical collaborators of the GnG-PK/PD-AD study. Pharmacogenetics and therapeutic drug monitoring of fluoxetine in a real-world setting: a PK/PD analysis of the influence of (non-)genetic factors. *(submitted for publication).*

III.2 REAL-WORLD CLINICAL CHARACTERIZATION OF SUBJECTS WITH DEPRESSION TREATED WITH ANTIDEPRESSANT DRUGS FOCUSED ON (NON-)GENETIC FACTORS, PHARMACOKINETICS AND CLINICAL OUTCOMES: *GnG-PK/PD-AD* STUDY

III.2 REAL-WORLD CLINICAL CHARACTERIZATION OF SUBJECTS WITH DEPRESSION TREATED WITH ANTIDEPRESSANT DRUGS FOCUSED ON (NON-) GENETIC FACTORS, PHARMACOKINETICS AND CLINICAL OUTCOMES: *GNG-PK/PD-AD STUDY*

III.2.1 INTRODUCTION

Depressive disorders are nowadays a global public health concern due to their high prevalence and poor results of the treatments currently available (14,16). Antidepressant medications alone or in combined therapy represent the main pharmacotherapeutic approach for the treatment of depression, with FLU, PAR and VEN among the most used antidepressant drugs worldwide (67,68,296). Nevertheless, clinical outcomes achieved with antidepressant medications have been widely variable and overall unsatisfactory, suggesting that drug therapy optimization/individualization is required. Those poor clinical outcomes have been attributed to genetic and non-genetic inter-individual differences and to the trial-error method that guides the pharmacotherapeutic interventions in this therapeutic area (68,69,97,98,203,225,226,228).

Over the last years, pharmacogenetics and therapeutic drug monitoring have converged in the investigation of personalized medicine and therapeutic biomarkers for antidepressant drug therapy, but without the expected success (65,69,70,87,167,176). Pharmacogenetic studies have essentially been focused on genetic factors, particularly those related to P-gp (*ABCB1* gene) and CYP isoenzymes, mainly CYP2C9, CYP2C19 and CYP2D6, once these proteins are key players in the bioavailability and biodisposition of antidepressant drugs (68,69,180,182,203,87,97,98,159,167,168,174,176). Nonetheless, human-drug interaction is not a simple gene-drug interaction; instead it is a complex, multigene and multifactorial one. In fact, in a real-world setting, pharmacokinetics, pharmacodynamics and clinical outcomes of antidepressant drugs are co-influenced not only by genetic, but also by non-genetic modulators, with emphasis on co-medication and co-morbidities (167,168,176). However, non-genetic/environmental factors and phenoconversion effects have been frequently underexplored at the level of the treatment of depression with antidepressant drugs, including in clinical pharmacogenetic studies (167,168,176,226,227). Such fact has been appointed as one of the main reasons

for the difficult in the identification of clinically useful biomarkers for the antidepressant drug treatments. Consequently, more holistic study approaches, considering individual genetic and non-genetic factors together, have been consensually recommended for future researches in the field of pharmacogenetics and personalized medicine (68,167,168,170,176).

Bearing in mind the aforementioned aspects, we designed and developed a real-world clinical study to investigate the impact of relevant genetic and non-genetic factors on the pharmacokinetics (plasma concentrations) and pharmacodynamics (clinical outcomes) of widely prescribed antidepressant drugs (FLU, PAR and VEN) in a naturalistic clinical setting of treatment of depression, focused on the identification of potential therapeutic biomarkers – the so-called *GnG-PK/PD-AD* study.

In this scope, the current work aimed to describe and characterize the *GnG-PK/PD-AD* study and its study population, for the first time, and to provide a real-world clinical characterization of subjects with depression treated with FLU, PAR and VEN, specifically in terms of relevant genetic and non-genetic individual factors, antidepressant pharmacokinetics and clinical outcomes. This will contribute to improve the clinical characterization and understanding of the state of art of the treatment of depression with FLU, PAR and VEN and to support further studies focused on the identification of potential therapeutic biomarkers for these antidepressant drugs. As highlighted above, there are scarce real-world data reported on literature about these matters, particularly integrating all these clinical variables and outcomes. To the best of our knowledge, this is the first real-world clinical report for the Portuguese population.

III.2.2 METHODS

III.2.2.1 STUDY DESIGN AND SUBJECTS

The *GnG-PK/PD-AD* study was a multicentre, cross-sectional, observational, real-world clinical study, integrating pharmacogenetics-pharmacokinetics-pharmacodynamics (clinical outcomes), that was carried out in Portugal and based on an Iberian Partnership. From January 2015 to June 2016, a multicentre recruitment of depressive outpatients treated with FLU or PAR or VEN on stable dosing regimen for at least two months was

performed in the naturalistic clinical setting of eight health units of the Centre Region of Portugal (see section III.1.). The exclusion criteria were: (a) patients under 18 years of age; (b) patients with dementia, autism, psychotic disorders, including bipolar disorder and eating disorders; (c) patients with significant physical or neurological disorders with mental repercussions and/or loss of consciousness and (d) patients unable to give an informed consent.

Eligible subjects were clinically and therapeutically characterized through a personal interview performed by a trained clinician. Relevant individual data were recorded: age, gender, ethnicity, target antidepressant drug (FLU or PAR or VEN) and corresponding dosing schedule, last dosing time, duration of the depressive episode, previously used antidepressant drug(s) and eventual reasons of its discontinuation, co-morbidities and other co-medication. Depression phenotype and remission were assessed by the HAMD and the presence of potential adverse effects by the ASEC (297,298). A venous blood sample (10 mL) was collected before the morning dose into EDTA-K_3 tubes for the quantification of plasma concentrations of the target antidepressant drugs and, when possible, of the respective main pharmacologically active metabolite (FLU + NFLU or PAR or VEN + ODV) and for genotyping of the *CYP2C9, CYP2C19, CYP2D6 and ABCB1* genes. No other medication adherence measures were considered.

The study and its protocols (Appendix A) were approved by a competent local Ethics Committee (*Ethics Committee of the Central Regional Health Administration*, Study: *Avaliação Farmacométrica do Impacto Clínico de Polimorfismos Genéticos do Citocromo P450 e da Glicoproteína-P na Farmacocinética e Farmacodinamia de Antidepressivos: Fluoxetina, Paroxetina e Venlafaxina*, study nº. 58/2014, Appendix B) and by the *Portuguese Data Protection Authority* (process nº. 8384/2014, authorisation nº. 9426/2014, Appendix B) and all participants provided an appropriate informed consent (Appendix A). Subjects who did not present clinical characterization data and a blood sample for drug quantification and genotyping were considered as subjects who did not complete the study protocol and, therefore, were not included in the study results.

III.2.2.2 GENOTYPING

Genetic analyses were performed at the Clinical Research Centre of the Extremadura University Hospital and Medical School (CICAB), employing previously described real-time and extra-long-polymerase chain reaction (RT-PCR and XL-PCR) methods for the screening of key SNPs associated with the alleles of interest (173,183,294,295). Table III.2.1 summarizes the allelic variants and/or SNPs investigated for the *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* genes.

Table III.2.1 Summary of the allelic variants and/or single nucleotide polymorphisms investigated for the *CYP2C9, CYP2C19*, *CYP2D6* and *ABCB1* genes. *ABCB1*, P-glycoprotein gene; CYP, cytochrome P450; RT-PCR, real-time polymerase chain reaction; SNPs, single nucleotide polymorphisms; extra-long polymerase chain reaction, XL-PCR.

Gene	Allelic variant	SNP(s)	rs code(s)	TaqMan® RT-PCR probe(s)
CYP2C9	*CYP2C9*2*	*430C>T*	rs1799853	C_25625805_10
	*CYP2C9*3*	*1075A>C*	rs1057910	C_27104892_10
	*CYP2C9*6*	*818delA*	rs9332131	C_32287221_20
CYP2C19	*CYP2C19*2*	*681G>A*	rs4244285	C_25986767_70
	*CYP2C19*3*	*636G>A*	rs4986893	C_27861809_10
	*CYP2C19*4*	*1A>G*	rs28399504	C_30634136_10
	*CYP2C19*5*	*1297C>T*	rs56337013	C_27861810_10
	*CYP2C19*17*	*806C>T*	rs12248560	C_469857_10
CYP2D6	*CYP2D6*2*	*1584C>G, 2850C>T*	rs1080385, rs16947	C_32407252_30, C_27102425_10
	*CYP2D6*3*	*2549del>A*	rs35742686	C_32407232_50
	*CYP2D6*4*	*1846G>A, 100 C>T*	rs3892097, rs1065852	C_27102431_D0, C_11484460_40
	*CYP2D6*5*	*whole-gene deletion (analysed by XL-PCR)*		
	*CYP2D6*6*	*1707delT*	rs5030655	C_32407243_20
	*CYP2D6*10*	*100C>T*	rs1065852	C_11484460_40
	*CYP2D6*17*	*1023C>T, 2850C>T*	rs28371706, rs16947	C_2222771_A0, C_27102425_10
	*CYP2D6*29*	*3183G>A*	rs59421388	C_34816113_20

Table III.2.1 Summary of the allelic variants and/or single nucleotide polymorphisms investigated for the *CYP2C9, CYP2C19*, *CYP2D6* and *ABCB1* genes. *ABCB1*, P-glycoprotein gene; CYP, cytochrome P450; RT-PCR, real-time polymerase chain reaction; SNPs, single nucleotide polymorphisms; extra-long polymerase chain reaction, XL-PCR.

Gene	Allelic variant	SNP(s)	rs code(s)	TaqMan® RT-PCR probe(s)
	*CYP2D6*35*	*1584 C>G, 31G>A*	rs1080385, rs769258	C_32407252_30, C_27102444_80
	*CYP2D6*41*	*2988G>A*	rs28371725	C_34816116_20
ABCB1		*1236C>T*	rs1128503	C_7586662_10
		2677G>T/A	rs2032582	C_11711720C_30 and C_11711720D_40
		3435C>T	rs1045642	C_7586657_20
		*rs*2032588		C_11711718_10

The genetic variants to be studied were the most relevant variants for the pharmacokinetics of FLU, PAR and VEN and for phenoconversion due to drug-drug interactions, considering those with the highest evidence level for gene x drug interaction (as determined by the Clinical Pharmacogenetics Implementation Consortium /PharmGKB) (86).

Briefly, genomic DNA was isolated using the QIAamp® DNA blood kit (QIAGEN, Hilden, Germany) and the presence of the target SNPs was investigated by RT-PCR, using commercially available TaqMan® probes, except for the *CYP2D6*5* allele. The presence of the *CYP2D6*5* allele and *CYP2D6* multiplied alleles was analysed by means of XL-PCR. Subjects positive for a multiplied allele or gene deletion were then analysed for gene copy number through RT-PCR. To discriminate between the *CYP2D6 (wild-type or *2) x N* and *(*4 or *10) x N* alleles, a 10-kb-long fragment was generated by XL-PCR from duplicated-positive subjects and thereafter tested for the respective SNPs by an established PCR restriction fragment length polymorphism approach (295). These XL-PCRs were performed in a Mastercycler 384 thermocycler (Eppendorf, AG, Hamburgo, Germany). RT-PCR genotyping was carried out with Taqman® assays, according to the manufacturer's instructions, including on specific primers and probes for these polymorphisms, and the Universal PCR Master Mix, No AmpErase UNG, which contains AmpliTaq Gold DNA

polymerase, dNTP, buffers, passive internal reference based on the ROX reference dye (Invitrogen, Carlsbad, CA, USA) and reaction conditions. All RT-PCR assays were conducted in 96-well plates, with each plate including negative (without DNA) and positive (heterozygous and/or homozygous) controls. Plates were read on an ABI 7300 instrument (Applied Biosystems). For *CYP2C9*, *CYP2C19* and *CYP2D6* genes, the wild-type allele (*1) was assigned when none of the alleles in study was detected.

III.2.2.3 THERAPEUTIC DRUG MONITORING

Steady-state trough plasma levels of FLU, NFLU, PAR, VEN and ODV were determined at the CICS-UBI, using two previously validated liquid chromatography methods (286,299), in accordance to the international accepted guidelines for therapeutic drug monitoring of antidepressant drugs (70).

III.2.2.4 PROCESSING AND INTERPRETATION OF DATA

III.2.2.4.1 CLINICAL AND THERAPEUTIC DATA

Patients were classified as young adults (18-24 years), adult (25-59 years) and elderly (≥ 60 years). Antidepressant daily dose was considered as the total prescribed daily dose. Depression was described as a chronic condition when duration was higher than 24 months (300). Co-morbidities were grouped taking into account the *ICD-10*. Drugs were categorized as antidepressants or antipsychotics or anxiolytics, hypnotics and sedatives or as other drugs, considering the *Anatomical Therapeutic Chemical System*.

Pharmacotherapeutic profiles were revised for substrates, inhibitors and inducers of the P-gp, CYP2C9, CYP219 and CYP2D6, using the *Transformer* (237) and *DrugBank* (79) databases, aiming to construct individual profiles of potential drug-induced phenoconversion for those proteins. Briefly, each recorded drug was individually classified regarding the potential of interaction with each protein. Then, based on the rationale of the AS for genetics of the CYP isoenzymes, Drugs-Protein Interaction Score (DPI) was herein created to represent the effects of a therapeutic profile on the functional activity of each protein (phenotype) into a numeric potential of

interaction/phenoconversion (potential of drug-induced phenoconversion) (182,183). This score consisted in the attribution of a numeric classification to the potential interaction effect of each drug, as shown below in Table III.2.2.

Table III.2.2 Properties of the Drugs-Protein Interaction Score: drug scoring and interpretation.

Drugs-Protein Interaction Score	
Type of drug	**DPI**
Substrate	- 0.25
Inhibitor	- 1
Inducer	1
DPI and CDPI	**Potential of drug-induced phenoconversion**
≤ -1	Potential to be inhibited
> - 1 to < 1	Low potential
≥ 1	Potential to be induced

CYP, cytochrome P450; DPI, drugs-protein interaction score; CDPI, combined CYP2C9-2C19-2D6 drugs-protein interaction score

The final value of the DPI for each protein resulted from the arithmetic sum of the classification of all the drugs of the therapeutic profile. For example, in a case of a patient under treatment with two substrates, one inhibitor and one inducer of the CYP2D6, the respective DPI would be:

CYP2D6 DPI = - 0.25 (substrate) - 0.25 (substrate) - 1.0 (inhibitor) + 1.0 (inducer) = - 0.5

Bearing in mind the combined CYP2D6-CYP2C9-CYP2C19 AS (CAS) for genetics (182,183), the combined CYP2D6-CYP2C9-CYP2C19 DPI (CDPI) was calculated as follows:

$$CDPI = (CYP2C9\ DPI + CYP2C19\ DPI + CYP2D6\ DPI)/3$$

Afterwards, subjects were classified according to different potential levels of drug-induced phenoconversion for P-gp, CYP2C9, CYP2C19, CYP2D6 and CYP2C9-2C19-2D6 system (Table III.2.2). At this level, it is important to bear in mind, that DPI/CDPI is a simplistic numerical codification/proposal, which was used in the *GnG-PK/PD-AD* study as a proof of concept to transform independent categorical variables (effect of each drug on

the functional activity of a protein) into a unique integrated continue variable (potential of drug-induced phenoconversion) and to allow a parametric statistical treatment of this variable. Its use in future studies will clarify its clinical relevance.

The presence of potential antidepressant-drug interactions was also investigated, considering the specific involvement of each protein in the pharmacokinetics of FLU, PAR and VEN. Specifically, the pharmacokinetics of VEN is dependent on the activity of P-gp, CYP2C9, CYP2C19 and CYP2D6, FLU is dependent on the activity of CYP2C9, CYP2C19 and CYP2D6 and PAR is dependent on the activity of P-gp, CYP2C19 and CYP2D6. Thus, a potential antidepressant-drug interaction was considered when the target antidepressant drug (FLU or PAR or VEN) was being co-administered with a drug potentially interacting (i.e. substrates, inhibitors and inducers) with these proteins specifically involved in their pharmacokinetics. Interactions were classified according to the nature of the interaction that the co-prescribed drug (precipitant) has on the target protein as follows: competition/inhibition, when the antidepressant drug was co-prescribed with a substrate or an inhibitor or a substrate-inhibitor; induction, for the co-prescription with an inducer or a substrate-inducer. When the precipitant drug was classified as inducer-inhibitor or as substrate-inhibitor-inducer, the nature of the interaction was considered as undetermined.

Regarding antidepressant clinical outcomes, HAMD score was considered as a measure of severity of depression and an indicator of remission, according to what has been used in previous cross-sectional studies with antidepressant drugs. Patients were classified as remitters if they reported a HAMD score ≤ 7 and as non-remitters if they reported a HAMD score ≥ 8 (159,301). Individual profile of adverse effects was characterized regarding severity by the Global Adverse Reaction Severity (ASEC-GARSI) and Positive Side-Effect Distress (ASEC-PSEDI) scores and regarding the number of adverse effects through Positive Side-Effect, Total (ASEC-PSET) and Positive Side-Effect, Relevant (ASEC-PSER) scores. ASEC-GARSI is the average score of the 21 items of the questionnaire and shows the overall level of severity of adverse effects. ASEC-PSEDI is the average score of the items scored above zero and a pure measure of intensity and also of the response style of the patient, i.e. whether the patient is “augmenting” or “attenuating” his/her adverse reactions. ASEC-PSET is the number of adverse reactions that are reported positive by the responder, while ASEC-PSER corresponds to the number of relevant

adverse effects, defined as those rated as moderate or severe and potentially treatment related (302).

III.2.2.4.2 GENETIC DATA

The frequencies of the *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* SNPs were investigated for the Hardy-Weinberg Equilibrium (HWE) and compared with those previously reported for healthy Caucasian populations also in HWE (172,184). As reported in previous works, genotypes of the *CYP2C9, CYPC19* and *CYP2D6* genes were translated in the AS, which was then used to determine the individual gPH for each isoenzyme: gPM or gIM or gEM or gUM (Table I.3.2) (182,183,303). Patients were classified accordingly to CAS, which was determined as below (Table I.3.2):

$$CAS = (CYP2C9\ AS + CYP2C19\ AS + CYP2D6\ AS)/3$$

Regarding the P-gp gene (*ABCB1*), haplotype analysis was carried out for the three most common *ABCB1* SNPs, *1236C>T, 2677G>T/A* and *3435C>*T, using PHASE, version 2.1 (304). Patients were classified into three haplotypes: haplotype 1 comprised patients homozygous for the wild-type haplotype (*CGC-CGC*); haplotype 2 was integrated by patients carriers of one copy of wild-type haplotype and a variant haplotype (*CGC-TTT, CGC-TGT, CGC-CAC, CGC-CGT, CGC-TAC, CGC-CTC, CGC-TGC*, and *CGC-TTC*); and haplotype 3 included patients with both copies of variant haplotypes (*TTT-TTT, TTT-CAT, TGC-TTT, CAC-TTT, CGT-TTT, CTC-TTC, CTT-TGT, TGT-TGT* and *CAT-TAT*).

III.2.2.4.3 PLASMA CONCENTRATIONS

Non-detected concentrations were treated as zero, while those concentrations below the limit of the quantification were defined as half of the lower limit of quantification of the method (i.e. 2.5 ng/mL for PAR, 5 ng/mL for VEN and 10 ng/mL for FLU, NFLU) (286,299).

III.2.2.5 DATA ANALYSIS

This work reports the primary data analysis of the *GnG-PK/PD-AD* study. All variables were characterized by means of adequate descriptive statistics. In order to better characterize the sample in study, relevant interactions between independent variables (genetic and non-genetic variables) were explored using bivariate statistical analysis, according to the purpose, distribution and scales of the variables. Associations and correlations between variables were tested using the Chi-squared test (χ^2) and the Spearman´s correlation (rs), respectively. In positive Chi-squared associations, Cramer's V and Eta values were considered as measures of the strength of association for nominal by nominal and nominal by interval variables, respectively. Only associations/correlations with a strength > 0.2 were reported. Differences were investigated by means of Mann Whitney U (U) and Kruskal-Wallis χ^2 with Dunn´s post-hoc analysis. HWE and comparison of the allele frequencies with the control groups was carried out using Chi-square test or an exact test. HWE analysis was implemented in the *RStudio* software (*version 3.3.2*), using the "HardyWeinberg" package (305). The impact of genetic and non-genetic factors (independent variables) on the pharmacokinetic and pharmacodynamic outcomes (dependent variables), i.e. independent-dependent variables interactions, were explored elsewhere. Data analysis was carried out with the *Statistical Package for the Social Sciences (SPSS, version 23.0, IBM Corp, 2015*). All p-values were two-tailed and statistical significance was set up at $p < 0.05$. Figure III.2.1 summarises the variables in study.

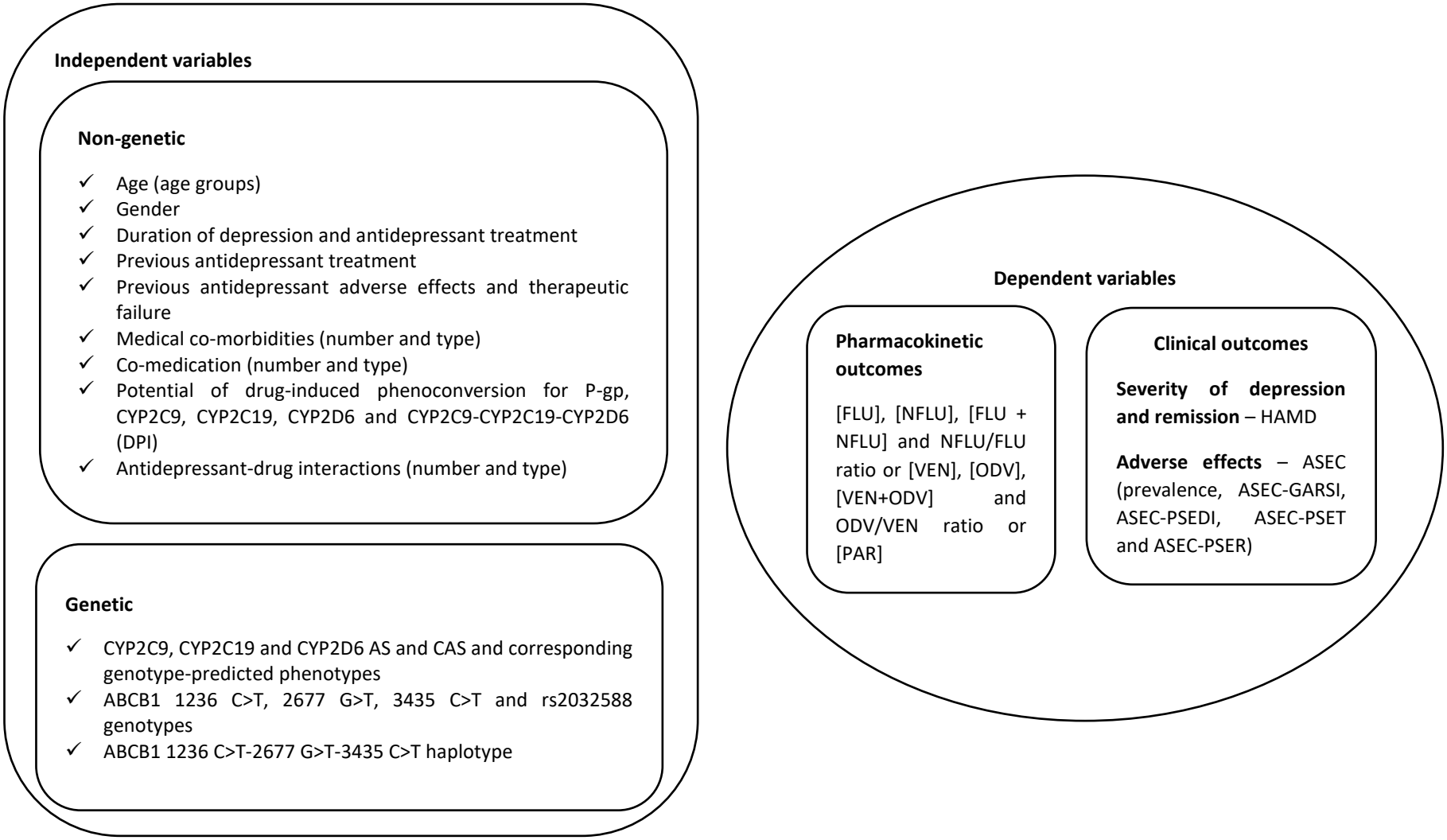


Figure III.2.1 Summary of the variables in study. [], plasma concentrations; ABCB1, P-glycoprotein gene; AS, activity score ASEC-GARSI, Global Adverse Reaction Severity; ASEC-PSEDI, Positive Side-Effect Distress; ASEC-PSET, Positive Side-Effect, Total; ASEC-PSER, Positive Side-Effect, Relevant; CAS, combined CYP2C9-CYP2C19-CYP2D6 activity score; CYP, cytochrome P450, DPI, drugs-protein interaction score; FLU, fluoxetine; HAMD, 17-item Hamilton rating score for depression; NFLU, norfluoxetine; ODV, *O*-desmethylvenlafaxine; PAR, paroxetine; VEN, venlafaxine.

III.2.3 RESULTS

III.2.3.1 CLINICAL AND THERAPEUTIC CHARACTERISTICS

A total of 184 depressed patients treated with FLU or PAR or VEN were recruited; however, 2 subjects did not complete the study protocol (no blood sample was available) and, therefore, only 182 were included in the study results. Table III.2.3 summarises the socio-demographic and clinical characteristics of the sample in study, including the details about the dosing regimens of the antidepressant drugs in study.

Table III.2.3 Socio-demographic and clinical characteristics of the sample in study (N = 182).

Variable (unit) (mean ± standard deviation) (minimum, median, maximum)	**Category**	**n (%)**
Age (years) (55.5 ± 12.2) (21, 56, 83)	Young adult (18-24)	4 (2.2)
	Adult (25-59)	107 (58.8)
	Elderly (≥60)	71 (39.0)
Gender	Female	149 (81.9)
	Male	33 (18.1)
Duration of the depressive episode (months)	2-6	6 (3.3)
	6-12	21 (11.5)
	12-24	21 (11.5)
	> 24	134 (73.6)
Antidepressant drug	Fluoxetine (FLU)	79 (43.4)
	Paroxetine (PAR)	31 (17.0)
	Venlafaxine (VEN)	72 (39.3)
Fluoxetine (FLU) daily dose (mg) (23.3 ± 8.1) (20, 20, 60)	20	67 (83.6) *
	40	11 (13.9) *
	60	1 (1.27) *
Paroxetine (PAR) daily dose (mg) (22.6 ± 6.8) (20, 20, 40)	20	27 (87.1) *
	40	4 (12.9) *

Table III.2.3 Socio-demographic and clinical characteristics of the sample in study (N = 182).

Variable (unit) (mean ± standard deviation) (minimum, median, maximum)	**Category**	**n (%)**
Venlafaxine (VEN) daily dose (mg) (108.9 ± 59.1) (37.5, 75, 300)	37.5	11 (15.3) *
	75	31 (43.1) *
	150	23 (31.9) *
	225	6 (8.3) *
	300	1 (1.4) *
FLU, PAR or VEN treatment duration (months)	2-6	32 (17.6)
	6-12	18 (9.9)
	12-24	34 (18.7)
	> 24	97 (53.3)
	NA	1 (0.5)
Previous antidepressant treatment	No	85 (46.7)
	Yes	97 (53.3)
	SSRI	46 (25.3)
	SNRI	7 (3.8)
	TCA	4 (2.2)
	TeCA	2 (1.1)
	Other	38 (20.9)
	Reason of discontinuation	
	Therapeutic failure	66 (36.3)
	Side-effects	15 (8.2)
	Therapeutic failure and side effects	6 (3.3)
	Other	10 (5.4)

n (%), absolute frequency (relative frequency); SSRI, selective serotonin reuptake inhibitor; SNRI, serotonin-noradrenaline reuptake inhibitor; TCA, tricyclic antidepressant; TeCA, tetracyclic antidepressant; NA, not available; * relative frequency calculated considering the number of subjects under treatment with the antidepressant drug in question.

All subjects were Caucasian, with more than 21 years old, and mostly females (81.9%). The data show that the majority of patients (73.6%) suffer from chronic depression (duration > 24 months) and were under treatment with FLU, PAR or VEN for at least 12 months (72%). More than half of the patients (53.3%) were previously treated

with other antidepressant drug(s), which were mainly discontinued due to therapeutic failure and/or side-effects.

Bivariate analysis found relevant statistical findings between the variables in study, as detailed in Table C.1. Specifically, men were more frequently under treatment with VEN, while women were more frequently under treatment with FLU. Moreover, female patients exhibited longer antidepressant treatment duration than male patients (median > 24 months *vs* 12-24 months, Table C.1). VEN was the antidepressant drug more frequently used after therapeutic failure with other antidepressants. Accordingly, 45 out of 72 (62.5%) subjects treated with VEN were previously treated with other antidepressant drugs. The antidepressant drug in study (FLU or PAR or VEN) was the first-line option for the treatment of the current depressive episode in 85 out of 182 patients (46.7%) and, as expected, the duration of the depressive episode was positively and strongly correlated with the duration of the antidepressant treatment (Table C.1). On the other hand, patients with chronic depression tended to keep the same antidepressant treatment for more than 24 months (Table C.1) and had been previously under treatment with other antidepressant drugs [75 out of 134 patients (56.0%)].

III.2.3.2 CO-MORBIDITIES AND PHARMACOTHERAPEUTIC PROFILE

Table III.2.4 summarizes the co-morbidities and pharmacotherapeutic profile of the sample in study. Further details can be found in Table C.2.

Table III.2.4 Summary of co-morbidities and pharmacotherapeutic profile of the sample in study (N = 182).

Category	**n (%)**	**Category**	**n (%)**
Medical co-morbidities (N = 396)		*Total of drugs (N = 862)*	
None	43 (23.1)	Monotherapy	21 (11.5)
1-2	70 (38.5)	2-4 drugs	77 (42.3)
3-5	63 (34.6)	> 4 drugs	84 (46.2)
> 5	6 (3.3)		

Table III.2.4 Summary of co-morbidities and pharmacotherapeutic profile of the sample in study (N = 182).

Category	**n (%)**	**Category**	**n (%)**
		Antidepressants (N = 234)	
Blood	2 (1.1)	1	134 (73.6)
Cardiovascular	97 (53.3)	2	44 (24.2)
Endocrinal, nutritional and metabolic	123 (67.6)	> 3	4 (2.2)
Eye and ear	9 (4.9)	*Antipsychotics (N = 34)*	
Gastrointestinal	24 (13.2)	0	152 (83.5)
Genitourinary	10 (5.5)	1	27 (14.8)
Infectious	3 (1.6)	> 2	3 (1.6)
Mental and behaviour	38 (20.9)	*Anxiolytics, sedatives or hypnotics (N = 104)*	
Musculoskeletal and connective tissue	39 (21.4)	0	88 (48.4)
Nervous system	15 (8.2)	1	84 (46.2)
Respiratory	13 (7.1)	2	10 (5.5)
Skin	4 (2.2)	*Other drugs (N = 490)*	
Others	19 (10.4)	0	45 (24.7)
		1-4	98 (53.8)
		> 4	39 (21.4)

n (%), absolute frequency (relative frequency)

Among the 182 patients, 139 (76.4%) presented at least one simultaneous disorder with depression and 161 (88.5%) were receiving at least one concomitant drug with the antidepressants in study. A total of 396 co-morbidities were recorded (2 co-morbidities per patient on average). Endocrinal, nutritional and metabolic, cardiovascular, and musculoskeletal diseases were the co-morbidities most frequently found, particularly hypertension, dyslipidaemia and diabetes *mellitus*. The number of co-morbidities increased as the age of patients increased, and, as expected, the elderly patients showed the highest prevalence of co-morbidities (Table C.1).

A total of 862 different drugs were registered. A great majority of patients were taking just one antidepressant drug (i.e. FLU or PAR or VEN), none antipsychotic and at least one or two anxiolytics, sedatives or hypnotics drug (Table III.2.4). Among the 48 patients treated with more than one antidepressant drug, 35 (72.9%) were using the antidepressant trazodone before bedtime for insomnia. Anxiety and insomnia disorders were underdiagnosed in our sample: 94 out of 182 patients (51.7%) were under treatment with anxiolytics, sedatives or hypnotics drugs, but just 17 out of 182 patients (9.3%) were diagnosed with such disorders (Table III.2.4 and Table C.2). The number of drugs recorded significantly increased with the number of co-morbidities and with the subjects' age. Lastly, patients who were using antidepressant drugs for the first time were significantly less administered with antipsychotic drugs than those patients who have previously been treated with antidepressant drugs (Table C.1).

III.2.3.3 POTENTIAL OF DRUG-INDUCED PHENOCONVERSION AND ANTIDEPRESSANT-DRUG INTERACTIONS

Among the 862 drugs recorded, 448 (52.0%), 414 (48.0%), 377 (43.7%) and 417 (48.4%) potentially interacted with P-gp, CYP2C9, CYP2C19 and CYP2D6, respectively. Most of the subjects (64.3-98.4%) were under risk of presenting P-gp, CYP2C9, CYP2C19 and CYP2D6 inhibited due to drug-induced phenoconversion (DPI). Sample distribution regarding the potential of drug-induced phenoconversion is provided in Table III.2.5.

Table III.2.5 Sample distribution regarding the potential of drug-induced phenoconversion (DPI).

Potential of drug-induced phenoconversion	**P-gp**	**CYP2C9**	**CYP2C19**	**CYP2D6**	**2C9-2C19-2D6***
Potential to be inhibited n (%)	117 (64.3)	153 (84.1)	134 (73.6)	179 (98.4)	163 (89.6)
Low potential n (%)	64 (35.2)	29 (15.9)	48 (26.4)	3 (1.6)	19 (10.4)
Potential to be induced n (%)	1 (0.5)	0 (0.0)	0 (0)	0 (0.0)	0 (0.0)
(C) DPI (mean ± SD)	-1.2 ± 1.1	-1.9 ± 1.3	-1.5 ± 1.0	-2.1 ± 1.1	-1.8 ± 0.9

n (%), absolute frequency (relative frequency); CYP, cytochrome P450; P-gp, P-glycoprotein; * combined CYP2C9-2C19-2D6 system (CDPI).

In addition, the potential of the P-gp and CYP2C9-CYP2C19-CYP2D6 systems to be inhibited by drug-induced phenoconversion significantly increased as the number of co-morbidities and administered drugs increased (Table C.1). Furthermore, subjects previously treated with antidepressant drugs significantly displayed a higher potential of the CYP2D6 to be inhibited by drug-induced phenoconversion than those who were using antidepressants for the first time (Table C.1). On the other hand, subjects with chronic depression presented a significantly higher potential of the CYP2C9 to be inhibited by drug-induced phenoconversion than subjects without chronic depression (Table C.1).

Regarding antidepressant-drug interactions, 147 out of 182 (80.8%) subjects were found to be at risk of occurrence of at least one interaction. A total of 793 potential antidepressant-drug interactions involving P-gp, CYP2C9, CYP2C19 and CYP2D6 were identified (Table III.2.6).

Table III.2.6 Sample distribution regarding the frequency of potential antidepressant-drug interactions at the level of P-gp, CYP2C9, CYP2C19 and CYP2D6.

Antidepressant- drug interactions	**n (%)** **N=793**
P-gp	
Competition/ inhibition	123 (15.4)
Induction	39 (4.9)
Undetermined	4 (0.5)
Total	166 (20.8)
CYP2C9	
Competition/ inhibition	188 (23.5)
Induction	1 (0.1)
Undetermined	8 (1.0)
Total	197 (24.7)
CYP2C19	
Competition/ inhibition	164 (20.5)
Induction	20 (2.5)
Undetermined	11 (1.4)
Total	195 (24.4)
CYP2D6	
Competition/ inhibition	204 (25.5)
Induction	24 (3.0)
Undetermined	7 (0.9)
Total	235 (29.4)

n (%), absolute frequency (relative frequency); CYP, cytochrome P450; P-gp, P-glycoprotein.

In this context, the number of potential antidepressant-drug interactions significantly increased with the number of co-morbidities and drugs that subjects were taking (Table C.1).

III.2.3.4 GENETICS

All the investigated SNPs were in HWE, with exception of *ABCB1 2677G>T/A* and rs2032588 *SNPs;* however, allele frequencies were similar to those described for Caucasian individuals (Table C.3). Specifically, 71 (39.0%), 90 (49.5%), 145 (79.7%) and 146 (80.2%) out of 182 patients displayed non-wild-type *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* genotypes, respectively. Table III.2.7 presents the frequency of the *CYP2C9, CYP2C19*, *CYP2D6* and *ABCB1* genotypes and *ABCB1 1236C>T-2677G>T-3435C>T* haplotypes observed in the sample in study.

Table III.2.7 Frequency of the *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* genotypes and *ABCB1 1236C>T-2677G>T-3435C>T* haplotypes in the sample in study (N = 182).

Genotypes	n (%)	Genotypes	n (%)
CYP2C9		***CYP2D6***	
**1/*1 wt*	111 (61.0)	**1/*1 wt*	37 (20.3)
**1/*2*	32 (17.6)	**1/*2*	26 (14.3)
**1/*3*	29 (15.9)	**1/*3*	1 (0.5)
**1/*6*	1 (0.5)	**1/*4*	28 (15.4)
**2/*3*	3 (1.6)	**1/*5*	5 (2.7)
**2/*2*	5 (2.7)	**1/*10*	1 (0.5)
**3/*3*	1 (0.5)	**1/*35*	7 (3.8)
CYP2C19		**1/*41*	20 (11.0)
**1/*1 wt*	92 (50.5)	**1/*2x2*	6 (3.3)
**1/*2*	26 (14.3)	**2/*2*	8 (4.4)
**1/*17*	43 (23.6)	**2/*3*	1 (0.5)
**2/*2*	4 (2.2)	**2/*4*	6 (3.3)
**2/*17*	10 (5.5)	**2/*5*	2 (1.1)
**17/*17*	7 (3.8)	**2/*6*	3 (1.6)

Genotypes	n (%)
CYP2D6	
**2/*41*	6 (3.3)
**2/*2x2*	1 (0.5)
**2x2/*35*	1 (0.5)
**2/*35*	1 (0.5)
**4/*4*	4 (2.2)
**4/*5*	1 (0.5)
**4/*6*	2 (1.1)
**4/*17*	1 (0.5)
**4/*35*	2 (1.1)
**4/*41*	3 (1.6)
**5/*17*	1 (0.5)
**6/*41*	1 (0.5)
**35/*35*	1 (0.5)
**35/*41*	1 (0.5)
**41/*41*	2 (1.1)
**10/*10*	1 (0.5)
**1x2/*4*	1 (0.5)
**1x3/*4*	1 (0.5)
ABCB1 1236C>T	
CC ^{wt}	60 (33.0)
CT	87 (47.8)
TT	35 (19.2)
ABCB1 2677G>T/A	
GG ^{wt}	65 (35.7)
GT	82 (45.1)
TT	32 (17.6)
GA	2 (1.1)
TA	1 (0.5)
ABCB1 3435C>T	
CC ^{wt}	59 (32.4)
CT	83 (45.6)
TT	40 (22.0)

Genotypes	n (%)
ABCB1 1236C>T-2677G>T-3435C>T	
haplotypes	
CGC-CGC ^{wt}	44 (24.2)
CGC-TTT	61 (33.5)
CGC-CGT	12 (6.6)
CGC-TGC	9 (4.9)
CGC-TTC	4 (2.2)
CGC-CTT	2 (1.1)
CGC-CAC	1 (0.5)
CGC-TAC	1 (0.5)
TTT-TTT	27 (14.8)
CGT-TTT	9 (4.9)
TGC-TTT	4 (2.2)
TTC-TTT	3 (1.6)
CTT-TTT	2 (1.1)
CGT-CTT	1 (0.5)
CTC-TAT	1 (0.5)
TGT-TTT	1 (0.5)
***ABCB1* rs2032588**	
GG ^{wt}	166 (91.2)
GA	13 (7.1)
AA	3 (1.6)

ABCB1, P-glycoprotein gene; CYP, cytochrome P450; n (%), absolute frequency (relative frequency), ^{wt}, wild-type genotype/haplotype

The functional activity of the CYP2C9, CYP2C19, CYP2D6 and of the combined CYP2C9-2C19-2D6 system was found to be genetically changed (gPH) in 71 (39.0%), 80 (44.0%), 101 (55.4%) and 143 (78.6%) out of 182 patients, respectively. Figure III.2.2 shows the sample distribution considering the gPH of those isoenzymes.

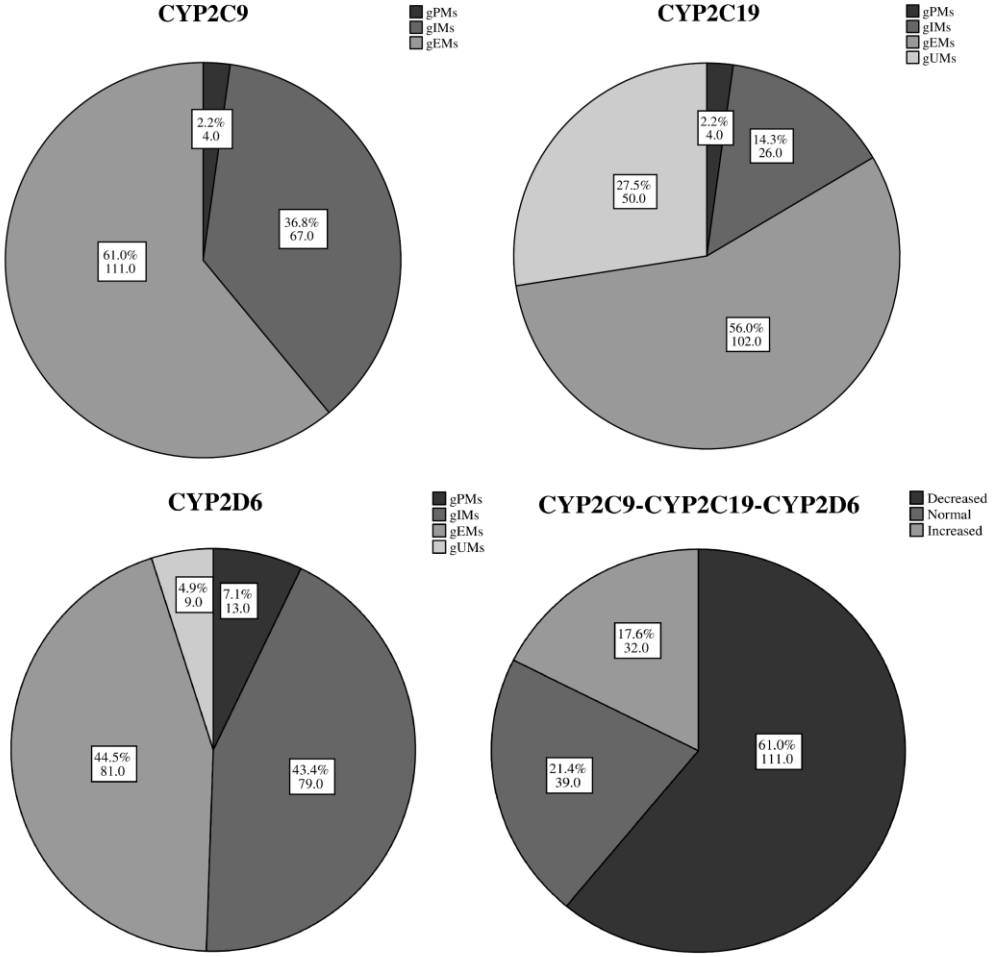


Figure III.2.2 CYP2C9, CYP2C19, CYP2D6 and combined CYP2C9-CYP2C19-CYP2D6 genotype-predicted phenotypes of the sample in study. Data are reported as relative and absolute frequencies. gEMs, genotype-predicted extensive metabolizers; gIM, genotype-predicted intermediate metabolizers; gPMs, genotype-predicted poor metabolizers; gUMs, genotype-predicted ultra-rapid metabolizers.

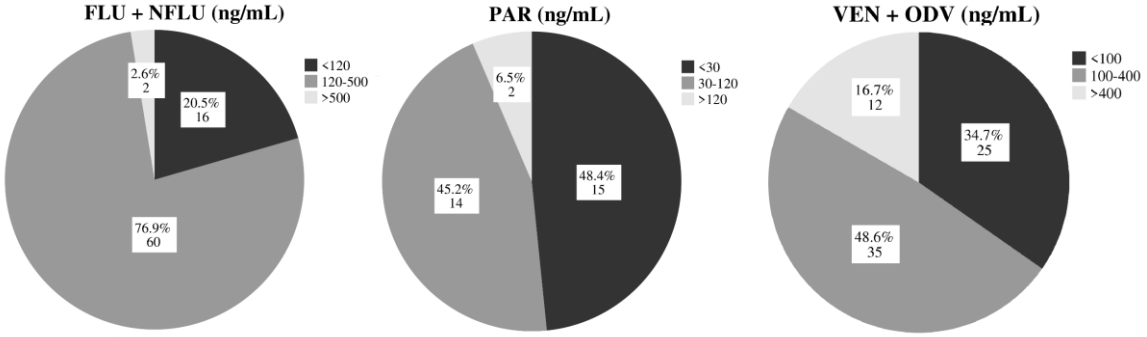
Regarding the *ABCB1* gene, 44 out of 182 (24.2%) patients were carriers of the wild-type haplotype 1, 100 out of 182 (54.9%) of the haplotype 2 and 38 out of 182 (20.9%) of the haplotype 3 (Table III.2.7). The *TTT* and *TTT-TTT* haplotypes were frequent in the present sample, with registration in 80 (44.0%) and 27 (14.8%) out of 182 patients,

respectively. Contrarily, the *ABCB1* rs2032588 *A*-allele was just identified in 16 out of 182 (8.8%) patients.

Lastly, patients with chronic depression presented a significantly higher CYP2C9 AS than individuals without chronic depression (Table C.1).

III.2.3.5 PLASMA CONCENTRATIONS AND CLINICAL OUTCOMES

Figure III.2.3 describes the steady-state plasma concentrations observed for the active portion of FLU (FLU + NFLU), PAR (PAR itself only) and VEN (VEN + ODV) and their distribution in relation to the recommended therapeutic range.



Active portion	FLU + NFLU (ng/mL)	PAR (ng/mL)	VEN + ODV (ng/mL)
Mean ± standard deviation	215.0 ± 135.7	50.1 ± 52.0 2	236.1 ± 258.2
Minimum, median, maximum	0.0, 197.7, 923.5	2.5, 32.7, 246.7	0.0, 156.7, 1373.2
Coefficient of variation, %	63.1	102.0	109.4
n	78*	31	72

Figure III.2.3 Steady-state plasma concentrations of the active portion of FLU, PAR and VEN for the sample in study (N = 181). Sample distribution regarding the plasma concentration classified in relation to the recommended therapeutic range (120-500 ng/mL for FLU + NFLU, 30 – 120 ng/mL for PAR and 100 – 400 ng/mL for VEN + ODV). FLU, Fluoxetine; NFLU, norfluoxetine; ODV, *O*-desmethylvenlafaxine; PAR, paroxetine; VEN, venlafaxine; * no FLU neither NFLU concentrations available for one subject due to interferences.

No plasma concentrations were detected in 6 out of 72 patients (8.3%) treated with VEN and 2 out of 79 patients (2.5%) treated with FLU, neither it was possible to

quantify the concentrations of 1 out of 79 patients (1.3 %) treated with FLU due to interferences. Concentrations of the active portion were below the recommended therapeutic range in 56 out of 182 (30.8%) patients and above in 16 out of 182 (8.8%). Noteworthy is the fact that 3 out of 72 (4.2%) patients treated with VEN, 1 out of 79 (1.3%) treated with FLU and 1 out of 31 (3.2%) treated with PAR displayed concentrations of the active portion equal or greater than two-fold of those recommended (VEN + ODV = 975.7, 1167.8 and 1373.2 ng/mL; FLU + NFLU = 923.5 ng/mL and PAR = 246.7 ng/mL).

Lastly, Table III.2.8 characterizes the sample by the antidepressant clinical outcomes with basis on the HAMD and the ASEC. More in-depth details about these assessments are provided in Table C.4 e Table C.5.

Table III.2.8 Characterization of the patient sample in study in terms of the antidepressant clinical outcomes evaluated by the *17-item Hamilton Depression Rating Scale* (HAMD) and the *Antidepressant Side-Effect Checklist* (ASEC) (N = 182).

HAMD assessment	**n (%)**
*HAMD score: 11.6 ± 7.0, 60.3 % (0.0, 11.0, 29.0) **	
Remission	
Remitters	61 (33.5)
Non-remitters	121 (66.5)
ASEC assessment	**n (%)**
*ASEC-GARSI: 0.76 ± 0.47, 61.8 % (0.0, 0.7, 2.4) **	
*ASEC-PSEDI: 1.85 ± 0.44, 23.8 % (1.0, 1.8, 3.0) **	
*ASEC-PSET: 8.39 ± 4.39, 52.3 % (0.0, 8.0, 21.0) **	
*ASEC-PSER: 2.09 ± 2.70, 129.2 % (0.0, 1.0, 16.0) **	
0	59 (32.4)
1	45 (24.7)
2 to 4	52 (28.6)
≥ 5	26 (14.3)

* mean ± standard deviation, % coefficient of variation (minimum, median, maximum)

III.2.4 DISCUSSION

The sample herein investigated was clinically and therapeutically framed with the known real-world scenario of treatment of depression. The majority of patients were females (21-59 years) with chronic depression and previously treated with other antidepressant drugs (Table III.2.3). Indeed, depression is two to three times more prevalent in women than in men and most common in the age group of 25-45 years (306,307). Noteworthy to mention is the fact that a gender bias favouring women has been reported in the diagnostic and treatment of psychiatric disorders, including on depression (307). Regarding to the overall clinical phenotype of depression based on the prevalence of depressive features identified by HAMD, the majority of patients (> 50%) registered depressed mood, insomnia, work incapacity, loss of interest and social dysfunction, anxiety, general somatic symptoms, such as loss of energy and fatigue, and genital symptoms, namely loss of sexual interest. Particularly worried is that 30.8% of the patients recorded suicide symptoms (Table C.4).

VEN was the drug most commonly used among the three target antidepressants (39.3%), mainly after therapeutic failure of other antidepressants (Table III.2.3 and Table C.1). This is explained by the fact that the sample herein tested is essentially composed by subjects with chronic depression, previously treated with other antidepressant drugs, and also because VEN is indicated in the treatment of resistant depression as an alternative to SSRIs (65,68). Furthermore, most patients were under antidepressant treatment for at least 12 months and the duration of treatment of patients suffering from chronic depression tended to be extended for more than 24 months (Table III.2.3 and Table C.1) Accordingly, the minimum recommended duration time for an antidepressant treatment trial is 6-12 months, being prolonged for at least 24 months in cases of chronic depression or risk of relapse. Gender bias was also herein observed regarding the duration of the antidepressant treatment, with females presenting longer treatment periods than males (Table C.1). Interestingly, males were more frequently treated with VEN while females with FLU (Table C.1). On the other hand, patients previously treated with antidepressant drugs were using more antipsychotics drugs than those patients who were being treated with antidepressant drugs for the first time (Table C.1). This finding results from the fact that the concomitant use of antidepressant and antipsychotic drugs is a

widely used augmentation strategy for situations of poor response or therapeutic failure (163).

Importantly, antidepressant treatments and clinical outcomes were noticeably suboptimal. High inter-individual variability was found in the clinical outcomes (CV%, 23.8 to 129.2%, Table III.2.8) and steady-state plasma concentrations (CV% ≥ 63.1%, Figure III.2.3) of FLU + NFLU, VEN + ODV and PAR. In agreement with previous data, more than half of the patients herein recruited did not achieve the remission of the depressive symptoms with the antidepressant treatment (66.5%) and/or registered at least one relevant antidepressant adverse effect (67.6%) (68,69). Each patient disclosed, on average, a total of 8 adverse effects, among which 2 were relevant (Table III.2.8, ASEC-PSET and PSER); an overall tendency for moderate severity was also observed (Table III.2.8, ASEC-GARSI and PSEDI). Worthy of note, dry mouth and problems with sexual function were the most frequently registered relevant adverse effects, i.e., those reported as related with the antidepressant drug and with at least moderate severity, followed by drowsiness and weight gain (Table C.5). Although plasma concentrations of antidepressant drugs may not be the ideal biomarker of antidepressant clinical outcomes, due to inconsistent correlations, therapeutic drug monitoring of FLU+NFLU, PAR and VEN+ODV has been recommended to better guide and optimize the treatments with these drugs (70). At this level, approximately 40% of all the patients presented concentrations outside of the recommended therapeutic range (Fig. 2). Despite this finding, low antidepressant daily doses were being administered in comparison to those that are approved for the treatment of depression (68,70). This reinforces the fact that in the real-world clinical setting antidepressant regimens have been highly variable, difficult to manage and, consequently, underexplored and therapeutic drug monitoring is a useful tool to optimize the antidepressant clinical outcomes.

Remarkably, the results herein presented showed that, besides the high genetic variability observed in the pharmacokinetics of antidepressant drugs (Figure III.2.2, Table III.2.7 and Table C.3), factors such as a co-morbid medical condition, polytherapy, a high risk of inhibition of P-gp, CYP2C9, CYP2C19 and CYP2D6 by drug-induced phenoconversion and, consequently, of drug-drug interactions may affected the pharmacokinetics/clinical outcomes of the target drugs (Table III.2.4, Table III.2.6 and Table III.2.8). These are non-genetic factors commonly present in the routine clinical practice of treatment of

depression and must be, hence, taken into account. To the best of our knowledge, this was the first time that a strategy to evaluate the potential of drug-induced phenoconversion (DPI) was employed (65,167,168,226). In fact, a high frequency of genetic polymorphisms and non-wild-type gPHs were observed for the C*YP2C9, CYP2C19, CYP2D6* and *ABCB1* genes (39-78.6%) (Figure III.2.2 and Table C.3).

Deviations from the HWE were found for the *ABCB1 2677G>T/A* and rs2032588 SNPs. On the other hand, the frequencies of all genetic variants under investigation were similar to those observed in the control groups (Table C.3). The same phenomenon was previously reported by our group, where the reliability of the genotyping method for the *ABCB1* gene was proven in relation to a reference method (173). Other studies have described similar outcomes for the *ABCB1* SNPs in the psychiatric field (308,309).

Overall, the genetic variations herein identified for CYP2C9 and CYP2D6 tended to lead to isoenzymes with defective functional capacity and, therefore, to gPMs and gIMs. Contrarily, a high prevalence of gUMs was registered for CYP2C19, as already reported (172). Consequently, most patients (61.0%) exhibited a genetically decreased combined CYP2C9-2C19-2D6 metabolic capacity (Figure III.2.2). For the *ABCB1* gene, a high frequency of the *TTT* and *TTT-TTT* haplotypes was verified. This finding deserves special attention, as previous associations have been documented between these haplotypes and clinical outcomes in psychiatry (87,173). In turn, the *ABCB1* rs2032588 *A*-allele was recently described by Bet *et al.* as a common genetic polymorphism associated with a better adverse effect profile to antidepressant drugs (310). Contrarily, this allele was scarcely identified within the present sample (< 10%, Table C.3).

Moreover, the most prevalent co-morbidities, namely hypertension, diabetes and musculoskeletal disorders, have known pathophysiological relationships with depression, explaining its frequent co-occurrence (232,307). Unsurprisingly, the number of co-morbidities and drugs that patients were taking increased as the age of patients increased and, in turn, the potential of drug-induced phenoconversion and of antidepressant-drug interactions also increased with the number of co-morbidities and of drugs (Table C.1). Thus, age, number of co-morbidities and of drugs are predictive risk factors for drug-induced phenoconversion and antidepressant-drug interactions at the pharmacokinetic level (CYP2C9, CYP2C19, CYP2D6 and P-gp).

Irrespective of the genetic background, the majority of the subjects in study were at risk of presenting the P-gp, CYP2C9, CYP2C19 and CYP2D6 inhibited due to drug-induced phenoconversion (Table III.2.5) and, consequently, at risk of occurrence of antidepressant-drug interactions (Table III.2.6). These findings are of utmost importance, because they show why pharmacogenetics and genotype alone has not been able to fully explain the drug outcomes variability and why it has been difficult to find clinically useful therapeutic biomarkers just based on genetics. As mentioned, pharmacogenetics research has been typically focused on the genetic factors, neglecting the impact of phenoconversion factors. However, in the real-world setting the actual phenotype of these pharmacokinetic-related proteins is co-modulated not only by genetic, but also by non-genetic and phenoconversion factors. In other words, the present study provides real-world-based evidence to change the mindset of pharmacogenetics and personalized medicine towards the integrated investigation of genetic and non-genetic factors and genotype-phenotype associations in the treatment of depression with antidepressant drugs, aiming the translation of the pharmacogenetics knowledge into clinical practice (65,167,168,226).

Of note, patients treated with previous antidepressant drugs (multiple antidepressant treatment) were at higher potential of the CYP2D6 to be inhibited by drug-induced phenoconversion, while patients with chronic depression showed a higher genetically determined functional activity and a high potential of the CYP2C9 to be inhibited by drug-induced phenoconversion (Table C.1). These are relevant issues, not only because they show the potential co-interaction between individual genetic and non-genetic factors in the definition of the real phenotype, but also because a poor antidepressant response has been found in patients with a decreased CYP2D6 functional activity. Such fact has been explained by the involvement of the CYP2D6 in the endogenous cerebral production of dopamine and serotonin in alternative pathways (68,311). Nonetheless, the study of the modulation of antidepressant response at the level of CYP2D6 has mainly been focused on the drug metabolism, without considering this endogenous impact (68,183,311). Thus, it is reasonable to conclude that the potential of the CYP2C9 and CYP2D6 to be inhibited by drug-induced phenoconversion may be clinically relevant non-genetic biomarkers for poor clinical outcomes (chronic depression and multiple antidepressant treatments) in the treatment of depression. At this level,

differences in the antidepressant response related with previous therapeutic failure and depression chronicity have been reported, but with no consensus (225,229). Further clinical evidence is needed on these matters.

In terms of study limitations, it is important to highlight that the present study should be interpreted as an exploratory work, due to its relatively small sample size and cross-sectional design. In fact, due to the naturalistic clinical setting of the study, the clinical assessments were performed at one single time point and no pre-treatment and follow-up data were available, which are frequently used to evaluate the clinical outcomes with antidepressant drugs. Moreover, real-world studies are tremendously complex and other non-studied factors, such as genetic factors related to pharmacodynamics, might impact the results. However, other clinical pharmacogenetic studies have used a cross-sectional approach to investigate the effect of genetic factors on the clinical outcomes of antidepressant drugs (159,301). On the other hand, the use of a "real world" sample increases the extrapolation capacity and representativeness of the study findings. Also, the power of the study is increased thanks to the homogeneity in terms of antidepressant treatment (only three antidepressants in study) and to the integrated study approach, involving the assessment of genetic and non-genetic factors.

Concluding, the work herein presented provides a real-world clinical characterization of the *GnG-PK/PD-AD* study population of depressive patients treated with FLU, PAR and VEN. Pharmacokinetic and clinical outcomes with FLU, PAR and VEN were found to be highly variable and suboptimal. Several genetic and non-genetic factors were identified as clinically relevant factors in the search for therapeutic biomarkers of these drugs, which deserve to be explored. Specifically, genetic polymorphisms, co-medication, co-morbidities and potential of phenoconversion and drug-drug interactions are key modulators of the pharmacokinetics and clinical outcomes and, therefore, must be studied together. The *GnG-PK/PD-AD* study sample demonstrated to be representative of the real-world clinical setting of the treatment of depression with FLU, PAR and VEN, constituting therefore a dataset with clinical validity for further analyses. In fact, this work constitutes the first data sub-analysis of the *GnG-PK/PD-AD* study. Further analyses will be carried out to specifically evaluate the co-integrated impact of genetic and non-genetic factors on the pharmacokinetics and pharmacodynamics of the antidepressant drugs in study. This work provides evidence to support and guide such future investigations.

III.3 PHARMACOGENETICS AND THERAPEUTIC DRUG MONITORING OF FLUOXETINE IN A REAL-WORLD SETTING: A PK/PD ANALYSIS OF THE INFLUENCE OF (NON-)GENETIC FACTORS

III.3 PHARMACOGENETICS AND THERAPEUTIC DRUG MONITORING OF FLUOXETINE IN A REAL-WORLD SETTING: A PK/PD ANALYSIS OF THE INFLUENCE OF (NON-)GENETIC FACTORS

III.3.1 INTRODUCTION

FLU is a SSRI and one of the first-line antidepressant drugs used in the treatment of depression. However, similarly to other antidepressant drugs, a wide inter-individual variability in the plasma concentrations and clinical outcomes has been observed with FLU (68,69,97,98,203,225,226,228).

Over the last years, pharmacogenetics and therapeutic drug monitoring have put efforts together to identify therapeutic biomarkers for antidepressant treatments, but with no practical success (68–70,87,160,167,171,176). Pharmacogenetic studies have been essentially centred on genetic factors, investigating binary associations between genotype or gPH and drug pharmacokinetics and/or pharmacodynamics in a non-real controlled setting. Rigid inclusion and exclusion criteria have been used to remove confusing effects, reducing the clinical value of those studies (68–70,87,160,167,171,176). At the level of antidepressant drugs, a special attention has been attributed to the genetic factors regarding P-gp (encoded by *ABCB1* gene) and the CYP2C9, CYP2C19 and CYP2D6 isoenzymes, once they mediate the pharmacokinetics of antidepressant drugs (68,87,167,168,176,182).

However, human-drug interaction is not a simple gene-drug relationship; instead it is complex, multigene and multifactorial interaction. In a real-world setting, drug concentrations and clinical outcomes are co-influenced not only by individual genetic characteristics, but also by non-genetic factors, with emphasis on co-medication and co-morbidities. Nonetheless, few studies have investigated the impact of these non-genetic and phenoconversion factors, namely drug-induced phenoconversion, despite the high risk that has been described in the clinical practice of treatment of depression due to polypharmacy (167,168,226,227). Phenoconversion is normally played by non-genetic factors, which change the genotype-phenotype relationship and, therefore, the genotype-drug clinical outcomes associations. Thus, the prediction of phenotype from genotype

(gPH) is inaccurate, as well as the study of the variability of drug outcomes only based on genotype and genetic factors (167,168,176,312).

This narrow view of pharmacogenetics has been the major responsible for the wide panacea of positive, but often conflicting results, hampering the identification of clinically useful biomarkers. In fact, no therapeutic biomarkers are used in the clinical practice for antidepressant drugs, including for FLU (69,97,98,159,174,180,203). FLU is mainly metabolized to NFLU by CYP2D6 and secondarily by CYP2C9 and CYP2C19. Several studies have analysed the effect of genetic polymorphisms of those isoenzymes on plasma levels of FLU and its active metabolite NFLU and/or on clinical outcomes; however, without real implications for the clinical practice (69,97,98,159,174,180,203). Moreover, in spite of the distinct genetic polymorphisms already identified for P-gp, its involvement in the pharmacokinetics of FLU is unclear and its influence on the drug outcomes has been scarcely studied (87,98). There is, hence, an undeniable unmet clinical need to improve the pharmacogenetics of antidepressant drugs, requiring the integration of genetic and non-genetic factors from the real-world clinical setting (68,87,167,168,176,227).

Thus, a real-world clinical study was performed to investigate together the impact of genetic and non-genetic factors on the pharmacokinetics and pharmacodynamics of widely prescribed antidepressant drugs, namely FLU, PAR and VEN, the so called *GnG-PK/PD-AD* study. The present work is focused on the treatment with FLU.

III.3.2 METHODS

III.3.2.1 SUBJECTS AND STUDY DESIGN

Outpatients diagnosed with depression, treated with FLU on stable dosing regimen for at least two months were recruited in the scope of the *GnG-PK/PD-AD* study. Patients were clinically and therapeutically characterized and submitted to therapeutic drug monitoring of FLU and NFLU and genotyping of *ABCB1, CYP2C9, CYP2C19* and *CYP2D6* genes. Antidepressant clinical outcomes, including remission and adverse effects were assessed by means of HAMD and ASEC, respectively (297,298,313). The clinical study design and protocol were described above in section III.2.2.

III.3.2.2 GENOTYPING

Genotyping was performed in the Clinical Research Centre of Extremadura University Hospital Medical School (CICAB), applying previously described and validated methods (173,183,294), as described in section III.2.2.2.

III.3.2.3 THERAPEUTIC DRUG MONITORING

Steady-state trough plasma levels of FLU and NFLU were determined at the CICS-UBI, using a previously validated liquid chromatography method (299), in accordance to the international accepted guidelines for therapeutic drug monitoring of antidepressant drugs (70).

III.3.2.4 PROCESSING AND INTERPRETATION OF DATA

III.3.2.4.1 CLINICAL AND THERAPEUTIC DATA

Patients were classified as young adults (18-24 years), adults (25-59 years) and elderly (≥ 60 years) (314,315). Antidepressant daily dose was considered as the total prescribed daily dose. Depression was described as chronic when duration was higher than 24 months (300). Co-morbidities were grouped taking into account the *ICD-10* (316).

Co-administered drugs were categorized as antidepressants or antipsychotics or anxiolytic, hypnotics and sedatives or as other drugs, considering the *Anatomical Therapeutic Chemical System* (317). Pharmacotherapeutic profiles were revised for substrates, inhibitors and inducers of the P-gp, CYP2C9, CYP219 and CYP2D6, using the *Transformer* (237) and *DrugBank* (79) databases, aiming to construct individual profiles of potential drug-induced phenoconversion for these proteins. Afterwards, DPI (potential of drug-induced phenoconversion) was calculated as described in section III.2.2.4.1. The combined CYP2D6-CYP2C9-CYP2C19 DPI for the specific FLU-NFLU metabolic pathway ($CDPI_{FLU\text{-}NFLU}$) was also calculated bearing in mind the approach of Villagra *et al*. for the AS (182):

$$DPI_{FLU\text{-}NFLU} = (DPI_{CYP2C9})\ 0.125 + (DPI_{CYP2C19})\ 0.125 + (DPI_{CYP2D6})\ 0.75$$

Subsequently, subjects were classified according to different potential levels of drug-induced phenoconversion for P-gp, CYP2C9, CYP2C19, CYP2D6 and FLU-NFLU metabolic pathway (section III.2.2.4.1.). The presence of potential antidepressant-drug interactions was also screened, considering the specific involvement of each protein in the pharmacokinetics of FLU (section III.2.2.4.1.).

Lastly, HAMD and ASEC metrics were used as described in section III.2.2.4.1. to characterise and assess the clinical outcomes of FLU, including remission and adverse effects.

III.3.2.4.2 GENETIC DATA

The frequencies of the *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* SNPs were investigated for the HWE and allele frequencies compared with those previously reported for healthy Caucasian populations in HWE (172,184).

Genotypes of the *CYP2C9, CYPC19* and *CYP2D6* genes were translated in the AS in accordance to previous works (182–184,303,318). The AS was then used to predict the metabolic phenotype from genotype (gPH) and classify each patient as gPM or gIM or gEM or gUM (Table I.3.2) (176). Due to the low frequency of gPMs, CYP2C9, CYP2C19 and CYP2D6 gPMs and gIMs were analysed alone and together and results were compared through a sensitivity analysis. Then, the combined CYP2C9-CYP2C19-CYP2D6 AS (CAS) for the FLU-NFLU metabolic pathway was calculated as presented below:

$$CAS_{FLU\text{-}NFLU} = (CYP2C9\ AS)\ 0.125 + (CYP2C19\ AS)\ 0.125 + (CYP2D6\ AS)\ 0.75$$

Subjects were classified into three groups characterized by having an increase, normal or decreased genotype-predicted metabolic capacity of the FLU-NFLU pathway (Table I.3.2) (182,183).

In relation to the *ABCB1* gene, a haplotype analysis was carried out for the three most common SNPs *(1236C>T, 2677G>T/A* and *3435C>*T) using PHASE, version 2.1 (304,319). Patients were then classified by the type of haplotype in relation to the presence of T allele. The *1236T-2677T-3435T* (*TTT*) haplotype was confirmed in those patients who carried at least one T variant in each one of the three *ABCB1* SNPs and the

1236TT-2677TT-3435TT (*TTT-TTT-TTT*) haplotype in those who were homozygous for the *T* allele in the three SNPs (173).

III.3.2.4.3 PLASMA CONCENTRATIONS

Non-detected concentrations of FLU and NFLU were treated as zero, while those concentrations below the limit of the quantification of the analytical technique (20 ng/mL) were treated as half of the lower limit of quantification of the method (10 ng/mL) (299). All concentrations were normalized by daily dose and, henceforward, concentrations will be referent to dose-adjusted concentrations.

III.3.2.5 DATA ANALYSIS

Figure III.2.1 summarises the study variables and data analysis. The impact of the genetic and non-genetic variables on the pharmacokinetics (plasma concentrations) and pharmacodynamics (clinical outcomes) of FLU was firstly screened by means of bivariate statistical analysis. FLU and NFLU plasma concentrations and antidepressant clinical outcomes based on the HAMD and ASEC related endpoints were considered as dependent variables, while genetic and non-genetic factors as independent variables. Plasma concentrations were then assumed as independent variables in the study of the relationship between concentrations and clinical outcomes.

Bivariate statistical tests were carried out according to the purpose, distribution and scales of the variables. Associations or correlations between variables were tested using the Chi-squared test (χ^2) or Fisher's Exact Test for counts below 5 and the Spearman´s correlation (rs), respectively. In positive Chi-squared/Fisher associations, Cramer's V and Eta values were considered as measures of the strength of association for nominal by nominal and nominal by interval variables, respectively. Only associations/correlations with a strength > 0.2 were reported. Differences were investigated by means of Mann Whitney U (U), Kruskal-Wallis χ^2 with Dunn´s post-hoc analysis and one-way ANOVA test (F). The normality of distribution of the variables was checked by the Shapiro-Wilks or Kolmogorov-Smirnov tests. The homogeneity of variances was evaluated by Levene's test for equality of variances when the one-way

ANOVA test was used. For this last one, Tukey post-hoc test was considered if the homogeneity of variances was fulfilled or the Welch ANOVA (Welch's F) and the Games-Howell post-hoc test if this assumption was violated.

Finally, a multivariate statistical analysis, using *Generalized Linear Models,* was performed for each dependent variable, including as predictors those variables that demonstrated statistical significance in the bivariate analysis, as well as other factors of clinical interest. Whenever possible, variables in numeric format were preferred. Overall, the models included age, gender, antidepressant daily dose, CYP2C9, CYP219, CYP2D6 AS and DPI as covariates. Models to predict clinical outcomes were adjusted to the number of antidepressant and antipsychotic drugs, duration of depression and of antidepressant treatment, number of co-morbidities and concentrations of the active portion (FLU+NFLU). Sensitivity analyses were performed for highly correlated significant variables (e.g. between CYP2D6 AS and CAS $_{FLU\text{-}NFLU}$), in order to select the best results in terms of model performance and clinical relevance, avoiding multicollinearity. *Post-hoc* pairwise comparison of estimated marginal means was used to compare categorical predictors. All statistical analyses were carried out with the *IBM SPSS Statistics v.2*3 (IBM Corp, 2015). All *p*-values were two-tailed and statistical significance was set up at $p < 0.05$.

III.3.3 RESULTS

A total of 79 Caucasian depressive patients treated with FLU were included in the current work. The mean ± standard deviation (minimum-maximum, median) age of the sample was 54.8 ± 12.1 years (21-83 years, 55 years), with only 2.5% aging between 18 and 25 years old, 63.3% between 26 and 59 years old and 34.2% with at least 60 years old. The majority of the subjects were women (92.4%) suffering from chronic depression (78.5%) and treated with FLU for at least 12 months (78.5%). Mean ± standard deviation (minimum-maximum, median) daily dose of FLU was 23.2 ± 8.3 mg (20-60 mg, 20 mg). Amongst the 79 patients, 38 (48.1%) had been previously treated with other antidepressant drug(s), which was/were discontinued mainly due to therapeutic failure and/or adverse effects (30 out of 38, 78.9 %). The great majority of patients were in a co-morbid (77.3%) and polypharmacy state (87.4%) and, consequently, under risk of suffering antidepressant-drug interactions at the pharmacokinetics level (70.9%).

Table III.3.1 Clinical and therapeutic characteristics of the sample treated with FLU (N = 79).

Variable/Category	n (%)
Nº of medical co-morbidities per patient	
0	18 (22.8)
1-2	21 (26.6)
3-5	36 (45.6)
>5	4 (5.1)
Type of medical co-morbidities	
Blood	2 (2.5)
Cardiovascular	40 (50.6)
Endocrinal, nutritional and metabolic	37 (46.8)
Eye and ear	6 (7.6)
Gastrointestinal	10 (12.7)
Genitourinary	8 (10.1)
Infectious	1 (1.3)
Mental and behaviour	10 (12.7)
Musculoskeletal and connective tissue	17 (21.5)
Nervous system	10 (12.7)
Respiratory	6 (7.6)
Skin	3 (3.8)
Others	3 (3.8)
Pharmacotherapeutic profile (total of drugs = 387)	
Monotherapy	10 (12.7)
2-4 drugs	27 (34.2)
≥ 5 drugs	42 (53.2)
Antidepressants (total = 99)	
1	60 (75.9)
2	18 (22.8)
> 3	1 (1.3)
Antipsychotics (total = 10)	
0	71 (89.9)
1	7 (8.9)
>2	1 (1.3)
Anxiolytics, sedatives or hypnotics (total = 35)	
0	45 (57.0)
1	33 (41.8)
2	1 (1.3)
Other drugs (total = 243)	
0	17 (21.5)
1-4	41 (51.9)
≥ 5	21 (26.6)
Potential antidepressant-drug interactions	
0	23 (29.1)
1-5	30 (38.0)
> 5	26 (32.9)
Remission	
Remitters	21 (26.6)
Non-remitters	58 (73.4)
HAMD score: 13.3 ± 7.1 (1.0, 14.0, 29.0) *	
ASEC-GARSI score: 0.9 ± 0.5 (0.0, 0.9, 2.0) *	
ASEC-PSEDI score: 1.9± 0.4 (1.0, 1.9, 2.8) *	
ASEC-PSET score: 9.3± 4.7 (0.0, 9.0, 21.0) *	
ASEC-PSER score: 2.3 ± 2.9 (0.0, 1.0, 13.0) *	

n (%), absolute frequency (relative frequency), * mean ± standard deviation (minimum, median, maximum)

Importantly, the majority of patients treated with FLU were at risk of inhibition of P-gp (86.1%), CYP2C9 (100%), CYP2C19 (93.7%), CYP2D6 (97.5%) and FLU-NFLU metabolic pathway (97.5%) by drug-induced phenoconversion. Table III.3.1 summarizes the main clinical and therapeutic characteristics of the sample treated with FLU.

Regarding genetic characteristics, frequencies of the SNPs in the population herein studied were in HWE, except those belonging to the *ABCB1 2677G>T/A* and rs2032588 SNPs. However, allele frequencies were not significantly different from those registered for other Caucasian population reported (Table C.3). The same phenomenon was also registered in other study from our research group involving the genetic analysis of the *ABCB1* gene, where the reliability of the genotyping method was proven in relation to a reference method (173). Additionally, other studies have described similar outcomes for *ABCB1* SNPs in the psychiatric field (308,309) and, therefore, those SNPs were not discarded of investigation.

CYP2C9, CYP2C19, CYP2D6 and *ABCB1* genes demonstrated to be highly polymorphic. The functional activity of the CYP2C9, CYP2C19 and CYP2D6 isoenzymes and of the FLU-NFLU metabolic pathway was found to be genetically changed (gPH) in 40.5%, 41.8%, 51.9% and 86.1% of the patients, respectively. Worthy of note is the fact that the majority of the CYP2C9 and CYP2D6 genetic polymorphisms were translated into gPM and gIM phenotypes, leading to a genetically decreased functional activity of the FLU-NFLU metabolic pathway.

Table III.3.2 presents the plasma concentrations of FLU, NFLU, FLU + NFLU and NFLU/FLU ratios by the ABCB1 1236 C>T, 2677 G>T/A, 3435 C>T and rs2032588 genotypes/haplotypes and by gPH of CYP2C9, CYP2C19, CYP2D6 and FLU-NFLU metabolic pathway.

Table III.3.2 Dose – adjusted plasma concentrations (ng/mL/mg) of FLU, NFLU, FLU + NFLU and NFLU/FLU ratios by the ABCB1 1236 C>T, 2677 G>T/A, 3435 C>T and rs2032588 genotypes/haplotypes and by the genotype-predicted phenotypes of CYP2C9, CYP2C19, CYP2D6 and FLU-NFLU metabolic pathway. Results are presented as mean ± standard deviation (median).

		FLU	NFLU	FLU+NFLU	n	NFLU/FLU	n
***ABCB1 1236 C>T* genotype**	*C/C*	4.2 ± 2.4 (4.0) *[b]	5.6 ± 2.9 (5.1)	9.8 ± 4.7 (9.4)	31	1.8 ± 1.5 (1.5)	31
	C/T	4.7 ± 2.8 (4.5) *[a]	5.3 ± 2.7 (4.8)	10.0 ± 3.8 (9.6)	32	1.9 ± 2.4 (1.3)	32
	T/T	2.9 ± 3.2 (2.2) *[ab]	4.2 ± 3.0 (3.6)	7.1 ± 6.0 (6.6)	15	2.5 ± 3.0 (1.6)	13
***ABCB1 2677 G>T/A* genotype**	*G/A*	3.7	8.6	12.3	1	2.3	1
	G/G	4.2 ± 2.4 (4.0)	5.4 ± 2.6 (4.6)	9.6 ± 4.5 (9.1)	31	2.0 ± 1.9 (1.3)	31
	G/T	4.6 ± 2.9 (4.5)	5.2 ± 3.1 (4.9)	9.8 ± 4.4 (9.9)	31	1.8 ± 2.1 (1.4)	30
	T/A	4.1	4.6	8.7	1	1.1	1
	T/T	3.2 ± 3.3 (2.4)	4.6 ± 3.0 (3.9)	7.8 ± 6.0 (6.7)	14	2.5 ± 3.0 (1.6)	13
***ABCB1 3435 C>T* genotype**	*C/C*	4.1 ± 2.5 (4.0)	5.7 ± 2.7 (5.2)	9.8 ± 4.5 (9.2)	30	2.1 ± 1.9 (1.6)	30
	C/T	4.1 ± 2.6 (3.7)	4.9 ± 3.2 (4.6)	9.0 ± 4.4 (9.1)	28	1.8 ± 2.2 (1.3)	26
	T/T	4.3 ± 3.5 (3.7)	4.9 ± 2.7 (4.6)	9.2 ± 5.6 (8.6)	20	2.0 ± 2.5 (1.4)	20
***ABCB1 rs2032588* genotype**	*A/A*	1.7	6.0	7.6	1	3.6	1
	G/A	5.3 ± 1.0 (5.3)	5.4 ± 1.3 (5.4)	10.7 ± 2.2 (10.7)	2	1.0 ± 0.1 (1.0)	2
	G/G	4.1 ± 2.8 (3.9)	5.2 ± 2.9 (4.6)	9.3 ± 4.8 (9.1)	75	2.0 ± 2.2 (1.4)	73

Table III.3.2 Dose – adjusted plasma concentrations (ng/mL/mg) of FLU, NFLU, FLU + NFLU and NFLU/FLU ratios by the ABCB1 1236 C>T, 2677 G>T/A, 3435 C>T and rs2032588 genotypes/haplotypes and by the genotype-predicted phenotypes of CYP2C9, CYP2C19, CYP2D6 and FLU-NFLU metabolic pathway. Results are presented as mean ± standard deviation (median).

		FLU	NFLU	FLU+NFLU	n	NFLU/FLU	n
***ABCB1 1236T-2677T-3435T* haplotype**	*non-TTT*	4.0 ± 2.4 (4.0)	5.5 ± 2.7 (4.8)	9.5 ± 4.5 (9.1)	36	2.0 ± 1.8 (1.5)	36
	TTT	4.6 ± 2.9 (4.5)	5.2 ± 3.1 (4.9)	9.8 ± 4.4 (9.9)	31	1.7 ± 2.1 (1.3)	29
	TTT-TTT	3.3 ± 3.6 (2.2)	4.5 ± 2.8 (3.6)	7.8 ± 6.1 (6.6)	11	2.6 ± 3.3 (1.6)	11
CYP2C9 gPH	gPMs	-	-	-	0	-	0
	gIMs	4.4 ± 2.7 (4.0)	5.3 ± 3.1 (5.1)	9.7 ± 4.7 (9.8)	31	1.7 ± 1.6 (1.0)	30
	gEMs	4.0 ± 2.8 (3.8)	5.2 ± 2.7 (4.6)	9.1 ± 4.7 (8.7)	47	2.1 ± 2.5 (1.5)	46
	gUMs	-	-	-	0	-	0
CYP2C19 gPH	gPMs	1.8	6.0	7.8	1	3.3	1
	gIMs	2.5 ± 2.1 (1.5)	3.1 ± 1.4 (3.3)	5.6 ± 3.2 (5.1)	9	1.8 ± 0.8 (2.2)	8
	gEMs	4.3 ± 2.8 (4.0)	5.7 ± 3.0 (6.0)	10.0 ± 4.8 (9.9)	45	2.1 ± 2.3 (1.4)	44
	gUMs	4.6 ± 2.9 (4.5)	5.0 ± 2.8 (4.6)	9.6 ± 4.6 (9.4)	23	1.7 ± 2.3 (1.3)	23
CYP2D6 gPH	gPMs	5.0 ± 3.3 (4.3)	2.0 ± 1.2 (2.1) *a	7.0 ± 3.8 (6.2)	4	0.5 ± 0.4 (0.5) *a	4
	gIMs	4.9 ± 3.1 (4.7)	4.7 ± 2.4 (4.5) *b	9.7 ± 5.0 (9.4)	33	1.2 ± 0.6 (1.0) *b	32
	gEMs	3.5 ± 2.4 (3.5)	5.6 ± 2.7 (5.8) *	9.1 ± 4.5 (8.9)	37	2.7 ± 2.8 (1.7) *ab	36

Table III.3.2 Dose – adjusted plasma concentrations (ng/mL/mg) of FLU, NFLU, FLU + NFLU and NFLU/FLU ratios by the ABCB1 1236 C>T, 2677 G>T/A, 3435 C>T and rs2032588 genotypes/haplotypes and by the genotype-predicted phenotypes of CYP2C9, CYP2C19, CYP2D6 and FLU-NFLU metabolic pathway. Results are presented as mean ± standard deviation (median).

		FLU	NFLU	FLU+NFLU	n	NFLU/FLU	n
FLU-NFLU gPH	gUMs	2.8 ± 1.2 (3.2)	8.7 ± 5.3 (8.1) *[ab]	11.4 ± 6.2 (11.5)	4	3.1 ± 1.2 (2.6) *	4
	Decreased	4.4 ± 3.0 (4.0)	4.5 ± 2.5 (4.3) *	8.9 ± 4.8 (8.8)	52	1.5 ± 1.3 (1.0) *[a]	50
	Normal	4.0 ± 2.8 (3.8)	6.5 ± 2.6 (6.1) *	10.4 ± 5.1 (9.3)	11	3.2 ± 3.7 (1.5) *	11
	Increased	3.5 ± 1.8 (3.4)	6.7 ± 3.3 (5.9) *	10.3 ± 4.0 (10.5)	15	2.7 ± 2.7 (2.1) *[a]	15

gpH, genotype-predicted phenotype; gEMs, genotype-predicted extensive metabolizers; gIMs, genotype-predicted intermedium metabolizers; gPMs, genotype-predicted poor metabolizers; gUMs, genotype-predicted ultra-rapid metabolizers.

* Overall statistically significant difference ($p < 0.05$) with Kruskal-Wallis or ANOVA test. Specific differences between groups found in post-hoc analysis, using Dunn´s test for Kruskal-Wallis and Tukey or Games-Howell tests for ANOVA, are identified with letters in superscript.

Pharmacokinetics (plasma concentrations) and pharmacodynamics (clinical outcomes) of FLU were highly variable between patients and displayed poor/suboptimal outcomes (Table III.3.1). Taking into account the plasma drug concentrations monitoring, no FLU neither NFLU plasma concentrations were detected in two patients; furthermore, it was not possible to determine the plasma concentrations of another patient due to sample interferences with the chromatographic peak of the analytes. Overall, mean ± standard deviation steady-state plasma concentrations of FLU, NFLU, FLU+NFLU and NFLU/FLU ratio were 4.1 ± 2.8, 5.2 ± 2.9, 9.4 ± 4.7 ng/mL/mg and 2.0 ± 2.2, respectively (CV%, 50.0-110.0 %). Concentrations of the active portion (FLU+NFLU) were below and above of the recommended therapeutic range (120-500 ng/mL) in 20.3% and 2.5% of the patients, respectively. In turn, 73.4% of patients did not achieve the remission of the depressive symptoms with FLU and 64.6% registered at least one relevant antidepressant adverse effect (Table III.3.1). Each patient disclosed, on average, a total of 9 adverse effects, among which 2 were relevant (Table III.3.1, ASEC-PSET and PSER); an overall tendency for mild-moderate severity was also observed (Table III.3.1, ASEC-GARSI and PSEDI).

III.3.3.1 BIVARIATE ANALYSIS

Multiple statistically relevant findings were identified between genetic and non-genetic factors and the antidepressant plasma concentrations and clinical outcomes. These interactions are presented in detail in Table C.6. At this level, it is worthy of note that no differences in the statistical outcomes were verified when the gPMs and gIMs were analysed together in the same group compared to the individual analysis. Furthermore, no significant relationships were found between drug plasma concentrations and clinical outcomes. This complex and multifactorial picture demanded the use of a multivariate approach for data analysis.

III.3.3.2 MULTIVARIATE ANALYSIS

III.3.3.2.1 IMPACT OF GENETIC AND NON-GENETIC FACTORS ON THE PLASMA CONCENTRATIONS

Four models were explored to explain and predict the concentrations of FLU, NFLU, FLU + NFLU and NFLU/FLU ratios, respectively. CYP2D6 AS was found as the only relevant predictor of the concentrations of FLU. For a unitary increment in the CYP2D6 AS (allele with normal function), concentrations of FLU decreased by an average of 19.7% (*B = - 0.197, OR = 0.821, p = 0.044*). In turn, the CYP2D6 AS and its gPH were also found as the only significant predictors of concentrations of NFLU and NFLU/FLU ratios. In detail, for a unitary increment in the CYP2D6 AS (allele with normal function), concentrations of NFLU and NFLU/FLU ratios increased by an average of 23.6% and 65.6%, respectively (*B = 0.236, OR = 1.266, p = 0.006 and B = 0.656, OR = 1.928, p < 0.001*). On the other hand, CYP2D6 gPM was found to be significantly associated with lower concentrations of NFLU and lower NFLU/FLU ratios compared to the other phenotypes (*B = - 1.162, OR = 0.313, p < 0.001 and B = - 1.807, OR=0.164, p < 0.001*). Pairwise comparisons showed that concentrations of NFLU and NFLU/FLU ratios were 2.0, 2.1 and 3.2-fold and 2.6, 5.3 and 6.1-fold significantly lower in gPM patients than in gIM, gEM and gUM patients, respectively (*p < 0.05*). Furthermore, CYP2D6 gIM phenotype was a significant predictor of the NFLU/FLU ratios (*B = - 0.839, OR = 0.432, p = 0.020*): pairwise comparisons showed ratios 2.0-fold lower in gIM patients than in gEM patients (*p < 0.05*). Also, $CAS_{FLU\text{-}NFLU}$ and gPH were significant predictors of the concentrations of NFLU and of the NFLU/FLU ratios, when tested as alternative to CYP2D6 AS and gPH; however, with inferior model performance. No statistically significant predictors were found for the concentrations of active portion (FLU+NFLU) (*p > 0.05*).

III.3.3.2.2 IMPACT OF GENETIC AND NON-GENETIC FACTORS ON THE CLINICAL OUTCOMES

Five models were investigated to explain and predict the severity and number of relevant adverse effects, as well as the severity of depression and remission, based on the ASEC-GARSI, ASEC-PSEDI, ASEC-PSER and HAMD scores.

The models for ASEC-GARSI and ASEC-PSEDI scores (severity of adverse effects) showed the *TTT*-haplotype and the presence of nervous system co-morbidities as significant predictors, as well as the potential of drug-induced phenoconversion for P-gp in the case of ASEC-GARSI. Patients with nervous system co-morbidities presented an ASEC-GARSI score 1.5-fold higher than patients with no nervous system co-morbidities (*B = 0.338, OR = 1.402, p = 0.035; pairwise comparison, p < 0.05*). In turn, carriers of the *TTT*-haplotype presented an ASEC-GARSI score 1.7 and 2.0-fold lower than carriers of the non-*TTT* and *TTT-TTT* haplotypes, respectively (*B = - 0.559, OR = 0.572, p < 0.05; pairwise comparison, p < 0.05*). Lastly, those patients with potential of the P-gp to be inhibited by drug-induced phenoconversion exhibited an ASEC-GARSI score 1.9-fold higher than those patients with low potential of phenoconversion (*B = 0.378, OR = 1.459, p = 0.015, pairwise comparison, p < 0.05*). Accordingly, patients with nervous system co-morbidities registered an ASEC-PSEDI score 1.2-fold higher than patients with no nervous co-morbidities (*B = 0.270, OR = 1.310, p = 0.049; pairwise comparison, p < 0.05*); while patients with *TTT*-haplotype presented an ASEC-PSEDI score 1.3 and 1.4-fold lower than those with non-*TTT* and *TTT-TTT* haplotypes, respectively (*B = - 0.423, OR = 0.655, p = 0.002; pairwise comparison, p < 0.05*).

In the model for ASEC-PSER score (number of relevant adverse effects) only the P-gp DPI was found as a relevant predictor. At this level, for each negative value of the P-gp DPI, there was an average increase of 100.9% in the ASEC-PSER score (*B = - 1.009, OR = 0.365, p = 0.043*). This suggests that there was approximately a duplication in the number of relevant adverse effects related to FLU with the co-administration of a P-gp inhibitor drug.

Analysing the results from the HAMD score model (which regards severity of depression), age, gender, duration of depression and the potential of drug-induced phenoconversion for CYP2D6 demonstrated to be relevant predictors. Specifically, there

was an average increase of 14.0% in the HAMD score for each unitary increment in the age of patients (*B = 0.140 OR = 1.150, p = 0.043*). Moreover, the HAMD score was 2.0-fold lower in male patients compared to females (*B = - 10416, OR = 2.994 x 10^{-5}, p = 0.001; pairwise comparison, p < 0.05*) and 2.5 and 2.2-fold higher in patients with a duration of depression of 2-6 months and 6-12 months compared to patients with chronic depression (*B = 13.084, OR = 4.812 x 10^{8}, p = 0.010 and B = 10.007, OR = 2.218 x 10^{7}, p = 0.013; pairwise comparison, p < 0.05*). Our model also showed that patients with potential of the CYP2D6 to be inhibited by drug-induced phenoconversion displayed a HAMD score 3.4-fold higher than patients with low potential (*B = 15.340, OR = 4.593 x 10^{9}, p = 0.029; pairwise comparison, p < 0.05*).

Finally, the model for the remission revealed gender and *TTT*-haplotype as significant predictors. Male patients presented a higher likelihood to be remitters than female patients (*B = 3,986, OR = 53.848, p = 0.036*). Likewise, carriers of the *TTT*-haplotype showed a higher likelihood to be remitters compared to the non-*TTT* and *TTT-TTT* haplotypes (*B = 1.986, OR = 7.283, p = 0.003 and B = 2.066, OR = 7.894, p = 0.025*).

III.3.4 DISCUSSION

The present work confirms the influence of CYP2D6 in the pharmacokinetics of FLU and provides evidence regarding the role of P-gp, which also seems to determine the drug pharmacokinetics and the pharmacological effects. Particularly important, this work reinforces the multifactorial and multigenic character of the pharmacokinetics and pharmacodynamics phenotype of FLU and signalized relevant individual genetic and non-genetic factors as potential therapeutic biomarkers in a real-world scenario (176,227). Table III.3.3 below summarizes the findings of this work.

Table III.3.3 Summary of the potential biomarkers of fluoxetine found in the real-world work herein reported.

Potential biomarker	**Pharmacokinetics/pharmacodynamics impact**
↑ CYP2D6 AS or CYP2D6 normal allele	↓ [FLU], ↑ [NFLU] and NFLU/FLU ratio
CYP2D6 gPM and gIM	↓ [NFLU] and NFLU/FLU ratio
Potential of the CYP2D6 to be inhibited by drug-induced phenoconversion	↑ severity of depression
TTT-haplotype	↓ severity of adverse effects
	↑ likelihood of remission
Potential of the P-gp to be inhibited by drug-induced phenoconversion	↑ severity of adverse effects
↓ P-gp DPI or P-gp inhibitor drug	↑ nº of relevant adverse effects
Nervous system co-morbidities	↑ severity of adverse effects
↑ Age	↑ severity of depression
Female gender	↑ severity of depression and ↓ likelihood of remission [1]
Chronic depression	↓ severity of depression [2]

[1] Overrepresentation of female patients, [2] Consequence of the study design

[], concentrations; ↑, increase; ↓, decrease; *ABCB1*, P-glycoprotein gene; AS, Activity Score; CYP, cytochrome P450; DPI, Drugs-Protein Interaction Score, FLU, fluoxetine; gEM, genotype-predicted extensive metabolizer phenotype; gIM, genotype-predicted intermedium metabolizer phenotype; gPM, genotype-predicted poor metabolizer phenotype; gUMs, genotype-predicted ultra-rapid metabolizer phenotype; NFLU, norfluoxetine; P-gp, P-glycoprotein.

With respect to concentrations, just the genetic background of CYP2D6 (AS and gPHs) was identified as a significant predictor. Overall, concentrations of FLU decreased and, consequently, concentrations of NFLU and the NFLU/FLU ratios increased as the genetically determined metabolic capacity of the CYP2D6 increased. CYP2D6 gPM and gIM were significant predictors of lower concentrations of NFLU and NFLU/FLU ratios (Table III.3.3).

No other genetic or non-genetic factors were identified as predictors of concentrations in the multivariate analysis. Nevertheless, it is consensual that CYP2C9 and CYP2C19 are also involved in the metabolism of FLU to NFLU, although in a lesser extent than CYP2D6 (160). On the other hand, the majority of the patients included in the present work were at a potential state of inhibition of the CYP2C9, CYP2C19 and CYP2D6 and, consequently, of the FLU-NFLU pathway due to drug-induced phenoconversion. This is in accordance with the results recently found by Preskorn *et al.* in the naturalistic clinical setting of treatment of depression (226). Consequently, concentrations of FLU tended to increase and concentrations of NFLU and NFLU/FLU ratios tended to decrease as the potential of inhibition increased (Table III.3.2). This suggests that, in a real-world practice, the role of the CYP2C9 and CYP2C19 in the metabolism of FLU is still lower due to the drug inhibitory effects aforementioned. Therefore, a clinically significant impact of any factor related with these isoenzymes is less probable to occur, mainly in polymedicated patients and potentially phenoconverted. To the best of our knowledge, this is the first work that objectively studied the effect of drug-induced phenoconversion on the FLU clinical outcomes (65,167,168,226). In line with these facts, most of the associations that have been reported between CYP metabolizing status and concentrations of FLU regards the CYP2D6 (69,98,147,160,180) and, even when positive results were found for CYP2C9 or CYPC19, no potential phenoconversion effects were considered (97,203). This raised another question in the present study: the potential of drug-induced phenoconversion of CYP2D6 did not reveal to be a predictor of concentrations of FLU in the multivariate analysis, probably because the phenoconversion of CYP isoenzymes also depends on the individual genetic background (168,176,227). Actually, gPM or gIM cannot be phenoconverted, at least with the same magnitude, as a gEM or gUM (168,176). A novel approach for the classification and analysis of CYP phenotypes assumes that gIMs, gEMs and gUMs patients who are taking potent inhibitors should be classified as phenoconverted poor metabolizers (168,176,227). However, based on the evidence available so far, we argue that there are patients that are not phenoconverted to an equivalent final poor metabolizer phenotype and that the co-interaction between genetics and non-genetics factors in a real-world setting results in more than four CYP phenotypes, with drug-induced phenoconversion playing a key role in this modulation (176). Thus, the future challenge that arises for the pharmacogenetics, including at the

level of the CYP isoenzymes, is the redefinition of the classic system of phenotype classification, in order to translate and incorporate genetic and non-genetic-induced phenoconversion interactions into a real phenotype. Our purposed model for the assessment of the potential of drug-induced phenoconversion (DPI) opens the door for this new paradigm along with the phenotyping tests (312).

Concordantly, the potential of the CYP2D6 to be inhibited by drug-induced phenoconversion was found as a significant predictor of poor clinical outcomes to FLU, particularly a higher severity of depression (Table III.3.3). This is compatible with the stereoselective metabolic and pharmacodynamic profile of FLU. While *S*- and *R*-FLU are almost equipotent in blocking serotonin reuptake, *S*-NFLU is 20-times more potent than *R*-NFLU. At this level, CYP2D6 is the major metabolizing isoenzyme of FLU and the main responsible for the formation of *S*-NFLU. During chronic treatments, the concentration of the *S*-enantiomers is about two times higher than the *R*-portion (97,98). Thus, in a scenario where the CYP2D6 functional activity is decreased, there is probably an alternative increase in the *R*-portion and in the less pharmacologically active *R*-NFLU, contributing to decrease the efficacy. Indeed, previous studies have observed lower levels of *S*-NFLU in patients with decreased functional activity of CYP2D6, but as far as we know, without demonstrating the clinical impact of this effect (97,98). Since no evaluation of the enantiomers was performed, this can help to explain why no impact on the concentrations of the global active portion (FLU+NFLU), neither a concentrations-response relationship was found in the present study. Furthermore, it has been described that the CYP2D6 enzyme catalyses the cerebral production of dopamine and serotonin by an alternative pathway and, therefore, can also modulate the antidepressant clinical outcomes at this level (311). Accordingly, poor antidepressant therapeutic response has been found in CYP2D6 gPMs (68).

Interestingly, genetic and non-genetic factors regarding P-gp revealed herein a relevant influence on the antidepressant clinical outcomes (Table III.3.3). Other studies have found positive associations between genetic variants of the *ABCB1* gene and FLU outcomes (87,98), but globally FLU has been deemed as a non-P-gp-substrate and this issue has not been investigated (87,98). Our study indicate that the *TTT*-haplotype seems to be favourable to better clinical outcomes with FLU, namely a higher likelihood of remission and a lower severity of adverse effects (Table III.3.3). This makes sense

considering that this allele has been linked to the increasing of the cerebral bioavailability of P-gp substrates. On this topic, recent evidence supports the *T*-allele in the *ABCB1 1236C>T, 2677G>T/A* and *3435C>T* SNPs as a better antidepressant response allele, but also as an allele responsible for the decreasing of tolerability, given that it has been associated with lower P-gp expression at the blood-brain barrier and easier access of P-gp substrate antidepressant drugs to the brain (87). This clarifies why, herein, the carriers of the *TTT*-haplotype presented better clinical outcomes than those with the *TTT-TTT* haplotype. Moreover, those investigations also explain why the *TTT-TTT* haplotype has been related with poor psychiatric outcomes, namely violent suicide attempts (173). Furthermore, the potential of the P-gp to be inhibited by drug-induced phenoconversion (DPI) predicted a worse tolerability profile, characterized by a higher severity of adverse effects and number of relevant adverse effects. Similarly to CYP2D6, *ABCB1* polymorphisms have been associated with mood disorders, including depression. Some authors have claimed that the decreased P-gp function at the blood–brain barrier may increase the accumulation of toxins in the brain, which may be involved, not only in the pathophysiology of mood disorders, but also in the poorer response to antidepressant drugs, regardless of whether they are substrates of the P-gp or not (98).

In turn, patients with co-morbidities affecting the nervous system demonstrated a higher severity of adverse effects, a logical fact since antidepressant drugs have several non-desired effects at the central nervous system and the occurrence of a potentiating effect is probable to occur. Moreover, the female gender and aging were both indicators of poor clinical outcomes with FLU (higher severity of depression and lower likelihood of remission). These factors have been consistently reported as relevant moderators of the antidepressant therapeutic response, with female patients older than 50 years demonstrating worse outcomes to SSRIs (225,229). Despite this, gender differences in the antidepressant therapeutic response remains a controversial topic, much in part due to the common gender bias favouring females (228,230,320). Once our study also presented a marked overrepresentation of female patients (92.4%), these results should be viewed with caution. Regarding patients with chronic depression, a lower severity of depression was predicted, but we considered this as a consequence of the longer antidepressant treatment. In truth, antidepressant therapeutic response has usually been inferior in patients with chronic depression (321).

To end, the results of this study should be interpreted as exploratory by its small sample size. Our findings are strengthened by the robust and controlled for confusing effects analysis, but real-world studies are tremendously complex and other non-studied factors might have influenced the results, such as genetic factors related to pharmacodynamics (69). Larger studies addressing these issues will be needed to confirm these results.

III.3.5 CONCLUSION

This study showed that the real-world treatment of depression with FLU presents significant genetic and non-genetic inter-individual variability and identified potential therapeutic biomarkers.

Genetically determined CYP2D6 activity was found to be a predictor of FLU and NFLU concentrations. In turn, genetic and non-genetic factors related to CYP2D6 and P-gp were found as potential biomarkers of the clinical outcomes of FLU. Specifically, the potential of the CYP2D6 to be inhibited by drug-induced phenoconversion was associated with a higher severity of depression. Moreover, *ABCB1 TTT*-haplotype was favourable to better clinical outcomes with FLU (higher likelihood of remission and lower severity of adverse effects). The potential of the P-gp to be inhibited by drug-induced phenoconversion was also related to a worse tolerability profile (higher severity and number of adverse effects). Lastly, the presence of nervous system co-morbidities was associated with a higher severity of adverse effects and aging and the female gender with a higher severity of depression and lower probability of remission.

CHAPTER IV

GENERAL DISCUSSION AND CONCLUSION

IV.1 GENERAL DISCUSSION

IV.1 GENERAL DISCUSSION

Depressive disorders are nowadays a serious public health concern, given its crescent prevalence, negative burden on societies and, particularly, its poor therapeutic outcomes. In fact, besides the large number of currently available antidepressant drugs, drug treatment of depression has deserved a special attention in the field of clinical pharmacology and pharmacotherapy, due to the high inter-individual variability in the antidepressant clinical outcomes (147,165,238–240). Over the last years, the acknowledgement that a significant portion of the inter-individual variability is associated with genetic factors has provided the impetus for the massive investigation of the impact of pharmacogenetic factors on the pharmacokinetics, pharmacodynamics and clinical outcomes of the antidepressant drugs. Nonetheless, pharmacogenetics alone has not been able to fully explain the antidepressant drug outcomes in a real-world setting and, therefore, the identification of clinically useful pharmacogenetic biomarkers for antidepressant drugs has not been as successful as it would be expected (68–70,87,160,167,171,176). In this context, the integrated use of pharmacogenetics and therapeutic drug monitoring, based on genotyping tests and drug plasma concentrations and considering clinically relevant genetic and non-genetic factors, has emerged as a promising way to optimize the treatment of depression with antidepressant drugs (167,181).

Thus, the work underlying this doctoral thesis consisted of a comprehensive and integrated pharmacometric evaluation of the clinical impact of genetic polymorphisms of the CYP2C9, CYP2C19, CYP2D6 and P-gp, as well as of non-genetic factors, on the pharmacokinetics and pharmacodynamics of widely used antidepressant drugs (FLU, PAR and VEN), aiming the identification of clinically relevant biomarkers for the treatment of depression with these drugs. For that purpose, a multicentre clinical study was planned and developed in the real-world setting of treatment of depression, exploring an integrated pharmacogenetics-pharmacokinetics-pharmacodynamics approach, the so called *GnG-PK/PD-AD* study. The present chapter is going to discuss the various topics addressed in the previous chapters in a holistic manner, providing a critical overview of the key matters of this research work in relation to the main goals proposed at the beginning of this thesis.

IV.1.1 RATIONALE AND STUDY DESIGN

The *GnG-PK/PD-AD* study was focused on FLU, PAR and VEN, since these are of the mostly used antidepressant drugs worldwide and, therefore, are drugs of extreme importance in the pharmacotherapy of depression. Similar to other antidepressant drugs, FLU, PAR and VEN have been associated to high inter-individual variability and poor clinical outcomes (147,159–161). On the other hand, these three drugs share relevant pharmacokinetic and pharmacodynamic features, such as the involvement of the same CYP isoenzymes in the metabolism, the potential involvement of P-gp in the pharmacokinetics and similar mechanisms of action, which allowed the use of a common clinical study design and protocol.

The *GnG-PK/PD-AD* study was conducted in the real-world clinical setting of the treatment of depression, with the aim to increase the clinical representativeness and the validity of the results and, consequently, to increase the potential of application into the clinical practice. An observational, multicentre, cross-sectional design with no follow-up period was chosen to be feasible and compatible with the operational constrains of a real-world clinical setting. Once the study was focused on the treatment of depression, patients with psychotic disorders, as well as with other disorders that could make impossible the application of the study protocol were not included, namely dementia, autism or other disorders with severe mental repercussions and/or loss of consciousness. Given the naturalistic scope of the study, no other restrictions were considered, namely at the level of the co-morbidities and co-medication. In a real-world setting, human-antidepressant drugs interaction is a complex, multigene and multifactorial interaction potentially co-influenced by individual genetic and non-genetic factors. Because of that, the *GnG-PK/PD-AD* study was planned as an integrated pharmacogenetics-pharmacokinetics-pharmacodynamics study, considering the impact of clinically relevant genetic and non-genetic factors together (167,168,226,227). A minimum of two months of continuous treatment with FLU or PAR or VEN on a stable regimen was required to safeguard that the drug was in steady-state conditions and had sufficient time to produce therapeutic effects. It is well-known that antidepressant drugs normally take 2-3 weeks to produce therapeutic effects (28,32,33).

Patients were clinically and therapeutically characterized through a personal interview performed by a trained clinician. Specifically, antidepressant clinical outcomes including remission and adverse effects were evaluated using scales duly validated for the effect: HAMD and ASEC, respectively (297,298). These scales have been used in the assessment of the clinical outcomes of antidepressant drugs by clinical studies with similar study design and purpose (159,301,302). At the pharmacogenetics level, CY*P2C9, CYP2C19, CYP2D6* and *ABCB1* were the genes selected for investigation because it is consensual that they are potential sources of genetic inter-individual variability in the drug outcomes of FLU, PAR and VEN (68,69,180,182,203,87,97,98,159,167,168,174,176). Specifically, the genetic polymorphisms to be studied were the most relevant variants for the pharmacokinetics of these drugs and for phenoconversion due to drug-drug interactions, considering those with the highest evidence level for gene x drug interaction (as determined by the Clinical Pharmacogenetics Implementation Consortium /PharmGKB database) (86). Apart from genetic factors, multiple other non-genetic factors are known to potentially affect the antidepressant drug outcomes, particularly co-medication and co-morbidities. These last two non-genetic factors have been described as the main causes of phenoconversion (167,168,176,312). Thus, the impact of clinically relevant non-genetic factors on the pharmacokinetics (drug plasma concentrations) and pharmacodynamics (clinical outcomes) of FLU, PAR and VEN was also investigated. Finally, steady-state trough plasma concentrations of the active portion of the antidepressant drugs in study (FLU+NFLU, PAR and VEN+ODV) were determined by previously validated bioanalytical methods and used as the classic biomarker of the antidepressant pharmacokinetic outcomes, following a therapeutic drug monitoring approach

IV.1.2 PHASE OF BIOANALYTICAL DEVELOPMENT

Bearing in mind that the *GnG-PK/PD-AD* study was based on a therapeutic drug monitoring approach, the first stage of this doctoral work was the development and validation of reliable bioanalytical tools for the quantification of FLU+NFLU, PAR and VEN+ODV. According to the international guidelines for therapeutic drug monitoring of antidepressant drugs, bioanalysis was focused on the pharmacologically active portion of each antidepressant drug (FLU+NFLU, PAR and VEN+ODV). Preliminary analyses found

significant differences in the bioanalytical behaviour and performance between these analytes, complicating the development of a simple and fast unique method suitable for the application in the real-world-clinical setting. Therefore, the development of two different HPLC methods was considered: a MEPS/HPLC-FLD method for the simultaneous quantification of VEN and ODV in human plasma and a MEPS/HPLC-FLD method for the quantification of FLU, NFLU and PAR in human plasma (Chapter II).

Both methods were successfully validated according to the international guidelines for validation of bioanalytical methods and demonstrated to be reliable, accurate and reproducible over a concentration range much wider than the usual therapeutic concentration range of the analytes (10-1000 ng/mL for VEN, 20-1000 ng/mL for ODV, 20-750 ng/mL for FLU and NFLU and 5-750 ng/mL for PAR), including when the dilution of a plasma sample is required due to concentrations that surpass the upper limit of quantification of the calibration range. Overall, accuracy and precision ranged between -10.3% to 16.7% and 0.1% to 13.6%, respectively (Chapter II).

Importantly, the storage and handling conditions of the authentic samples of the *GnG-PK/PD-AD* study were set up in this phase through dedicated stability studies at several relevant conditions. Specifically, the analytes in study (FLU, NFLU, PAR, VEN and ODV) demonstrated to be stable in human plasma at room temperature for 4 h, at 4 ºC for 24 h, after three freeze-thaw cycles at -20 ºC and at -20 ºC for 30 days and in processed plasma samples at room temperature for 12 h. Considering these conditions, the MEPS/HPLC-FLD assays were then successfully used to quantify FLU, NFLU, PAR, VEN and ODV in plasma samples collected from depressed patients and recruited in the scope of the *GnG-PK/PD-AD* study. The bioanalytical performance of the two methods in the quantification of authentic samples was similar to that documented in the development and validation phase, attesting the validity and usefulness of the methods in the real-world clinical practice.

Globally, these two methods display several important analytical advantages in relation to the majority of the methods described in literature for these analytes. First, these methods require a low volume of plasma sample (100-500 µL), enabling the use of less invasive sampling collection procedures and additional analyses from the same sample if necessary. Second, MEPS is a miniaturized, reusable, low-cost technology for sample preparation. The bioanalytical protocols herein developed allow the use of the

same MEPS cartridge for more than 100 analyses. Numerous HPLC methods (243,244,253–256,271–273,276–278,245,279–285,246–252) are available in literature for the quantification of FLU, NFLU, PAR, VEN and ODV in human plasma and serum; however, most of them are based on classic sample extraction procedures, namely LLE and SPE (244,247,277,281–285,248,250–254,257,271). However, one of the main current trends in bioanalysis is the use of miniaturized devices for sample preparation, such as MEPS (258,278,286–291). Then, the chromatographic and the sample preparation procedures of these methods are quite simple and fast (about 30 minutes per analysis) and require less expensive technology, normally available in the majority of bioanalytical laboratories, translating in high-throughput, high cost-effectiveness and translational analyses. Lastly, these methods enable the quantification of the active portion of the drug, the relevant portion for the pharmacological effects, a common pre-requisite for pharmacokinetic and therapeutic drug monitoring studies involving these drugs.

Thus, these bioanalytical assays are attractive and promising tools for the routine therapeutic drug monitoring, as well as for other clinical pharmacokinetic and toxicological-based studies (70,98,279).

IV.1.3 THE *GnG-PK/PD-AD* STUDY

The *GnG-PK/PD-AD* study was the main focus of the present doctoral work. All the phases of the study were successfully carried out and completed, specifically:

- the clinical phase, where 182 patients with depression under treatment with FLU, PAR or VEN were recruited and clinically characterised according to the study protocol;
- the bioanalytical phase, where the steady-state trough plasma concentrations of drug/metabolite (FLU+NFLU, VEN+ODV and PAR) of the patients in the study were determined, using the methods validated in the previous phase of bioanalytical development;
- the genotyping phase, where the *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* genes of the patients in study were genotyped, using methods already validated;
- the phase of pharmacometric analysis, where the data were integrated and analysed, considering the two main study objectives.

The first objective of the *GnG-PK/PD-AD* study was to provide a real-world clinical characterization of Portuguese depressive patients treated with FLU, PAR and VEN, specifically in terms of pharmacokinetic (drug plasma concentrations) and pharmacodynamic outcomes (clinical outcomes) and clinically relevant genetic and non-genetic individual factors. This was carried out in the first work of the *GnG-PK/PD-AD* study (chapter III, section III.2), which resulted in a publication of a full article in a peer-reviewed international journal.

The first key finding of the *GnG-PK/PD-AD* study was that, in line with previous studies, FLU, PAR and VEN are frequently associated to high inter-individual variability and poor pharmacokinetic (drug plasma concentrations) and pharmacodynamic (clinical outcomes) outcomes in the real-world clinical setting of treatment of depression. At the pharmacokinetics level, steady-state trough plasma concentrations of FLU + NFLU, VEN + ODV and PAR were found to be highly variable between patients (CV%, 63.1-109.4), with approximately 40% of the sample registering concentrations outside of the recommended therapeutic range. However, low antidepressant daily doses were being administered in comparison to those that are approved for the treatment of depression with these drugs (68,70). In terms of clinical outcomes, more than half of the patients did not achieve the remission of the depressive symptoms with the antidepressant treatment and/or registered at least one relevant antidepressant adverse effect (68,69). The majority of patients (> 50%) registered depressed mood, insomnia, work incapacity, loss of interest and social dysfunction, anxiety, general somatic symptoms, such as loss of energy and fatigue, and genital symptoms, namely loss of sexual interest. Indeed, depression substantially impairs the individual's ability to function at work or school or cope with daily life, representing the leading cause of disability and the major contributor to the non-fatal health loss and disease burden worldwide (17,19,20). On the other hand, each patient disclosed, on average, a total of 8 adverse effects, among which 2 were relevant; an overall tendency for moderate severity was also observed. In agreement with the literature, dry mouth and problems with sexual function were the most frequently registered relevant adverse effects, followed by drowsiness and weight gain (chapter III, section III.2). Actually, SSRIs and SNRIs overall share a common adverse effects profile, with weight gain, sleep disturbance and sexual dysfunction being the most problematic and reported adverse effects with these drugs (71). In turn, one of the main concerns in

relation to depression is the associated risk of suicide. Approximately 800 thousand people die worldwide due to suicide every year, being estimated that up to half of these suicides occur within a depressive episode. Accordingly, around 31% of the patients were identified with suicide symptoms in the *GnG-PK/PD-AD* study (chapter III, section III.2). In fact, patients with depression display a risk of suicide more than 20-fold greater than the general population (15–18). On this matter, SSRIs have been associated with a potential increase in suicide ideation and in the rate of suicide attempts. However, this is not completely consensual, since these symptoms may also be a direct consequence of the psychiatric disorder (63,71,77,93,94). On the other hand, the poor clinical outcomes with the antidepressant treatments may be a difficult and frustrating experience for depressive patients and, consequently, may increase the risk of non-compliance and even of suicide (71,162).

The second key finding of the *GnG-PK/PD-AD* study was a high genetic and non-genetic inter-individual variability in the real-world clinical setting of treatment of depression, potentially linked to the poor clinical outcomes observed for FLU, PAR and VEN. Several genetic and non-genetic factors were identified as potential sources of inter-individual variability and poor clinical outcomes with the antidepressant drugs in study. Specifically, a high frequency of genetic polymorphisms and non-wild-type gPHs were found for the C*YP2C9, CYP2C19, CYP2D6* and *ABCB1* genes (39-78.6%). Apart from these genetic factors, a co-morbid medical condition, polytherapy, a high risk of inhibition of P-gp, CYP2C9, CYP2C19 and CYP2D6 by drug-induced phenoconversion and, consequently, of drug-drug interactions were also found (Chapter III, section III.2). Such findings are of utmost importance, because they show why pharmacogenetics and genotype alone has not been able to fully explain the variability in antidepressant drug outcomes and why it has been difficult to find clinically useful therapeutic biomarkers just based on genetics for these drugs. As previously discussed, pharmacogenetics research has been typically focused on the genetic factors and few studies have investigated the impact of non-genetic and phenoconversion factors. However, in the real-world clinical setting, the pharmacokinetics and pharmacodynamics is co-modulated not only by genetic, but also by non-genetic and phenoconversion factors. Thus, one of the main insights of the *GnG-PK/PD-AD* study is that the impact of genetic and non-genetic factors on the clinical outcomes of antidepressant drugs must be studied together. Moreover, these findings

reinforce the thesis that the antidepressant treatments with FLU, PAR and VEN are highly variable, difficult to manage and, consequently, suboptimal and that the integrated use of pharmacogenetics and therapeutic drug monitoring constitutes a useful way to optimize the antidepressant clinical outcomes.

In summary, the first work of the *GnG-PK/PD-AD* study provided useful real-world epidemiological, pathophysiological and therapeutic data for the clinical practice of treatment of depression, including but not limited to the clinical phenotype and symptomatology of depression and pattern of prescription, efficacy, safety and tolerability data for FLU, PAR and VEN. Overall, the *GnG-PK/PD-AD* study sample was found to be clinically and therapeutically framed with the described real-world clinical scenario of treatment of depression (65,68,163,306,307). This work demonstrated that the *GnG-PK/PD-AD* study sample is an adequate dataset for further analyses and provide evidence to support and guide the investigations planned in the second objective of study. Several works have reported a high inter-individual variability in the clinical outcomes of antidepressant drugs. However, few have provided a so detailed clinical picture of the real-world treatment of depression, considering individual genetic and non-genetic factors together. Particularly, the majority of the pharmacogenetic studies have not considered the impact of potential phenoconversion effects, such as those potentially induced by co-medication and drug-drug interactions (69,87,167–171,176,226,227,95,123,147,160,163–166). Contrarily, in the *GnG-PK/PD-AD* study, a system to evaluate the potential of drug-induced phenoconversion was proposed and applied to characterise the study sample. To the best of our knowledge, this was the first time that the potential of drug-induced phenoconversion was objectively and quantitatively addressed and characterised. Remarkably, this study with this framework is unique in Portuguese depressive patients and, as far as we know, it is the first genetic characterization of the CYP2C9, CYP2C19, CYP2D6 and P-gp for this population.

The second objective of the *GnG-PK/PD-AD* study was to evaluate the impact of clinically relevant genetic and non-genetic factors, identified in the first work, on the pharmacokinetics (drug plasma concentrations) and pharmacodynamics (clinical outcomes) of the antidepressant drugs in study. Given the specific pharmacokinetics/pharmacodynamics profile of each drug in study, this second objective needed to be addressed per antidepressant drug. Among the three antidepressant drugs,

FLU is the mostly used drug for the treatment of depression and, accordingly, it was that with a larger sample size in the *GnG-PK/PD-AD* study. Thus, FLU was the first drug to be individually studied and the only one at the time of this Thesis. Such specific pharmacometric analysis for FLU was reported in the second work of the *GnG-PK/PD-AD* study, which is currently under publication process (Chapter III, section III.3).

This work provided several key findings for the clinical pharmacology and pharmacotherapy of depression with FLU. Overall, this second work confirmed the influence of the CYP2D6 in the pharmacokinetics of FLU, but particularly, provided evidence regarding the role of P-gp, which also appears to determine the drug pharmacokinetics and the pharmacological effects. Moreover, this work reinforced the multifactorial and multigenic character of the pharmacokinetics and pharmacodynamics phenotype of FLU and signalized relevant individual genetic and non-genetic factors as potential therapeutic biomarkers in a real-world scenario.

Specifically, genetically determined CYP2D6 activity was found to be a predictor of FLU and NFLU concentrations. Overall, concentrations of FLU decreased and, consequently, concentrations of NFLU and the NFLU/FLU ratios increased as the genetically determined metabolic capacity of the CYP2D6 increased. In agreement, CYP2D6 gPM and gIM were found as significant predictors of lower concentrations of NFLU and NFLU/FLU ratios. On the other hand, despite being involved in the metabolism of FLU, CYP2C9 and CYP2C19 did not demonstrate to be relevant biomarkers of the FLU concentrations, neither of the clinical outcomes. This was explained by the fact that CYP2C9 and CYP2C19 to be minor metabolizing isoenzymes of FLU and, as discussed above, by these isoenzymes to be potentially inhibited due to drug-induced phenoconversion in the real-world clinical setting of treatment of depression, decreasing still more its metabolic role in the FLU-NFLU pathway. Importantly, these findings are aligned with the published literature at this level. In fact, the majority of the associations reported between CYP metabolizing status and concentrations of FLU are related to the CYP2D6 (69,98,147,160,180) and, even when positive results were found for CYP2C9 or CYPC19, no potential phenoconversion effects were considered (97,203).

In turn, genetic and non-genetic factors related to CYP2D6 and P-gp were found as potential biomarkers of the clinical outcomes of FLU. Particularly, the potential of the CYP2D6 to be inhibited by drug-induced phenoconversion was associated with a higher

severity of depression. This was justified by the well-described stereoselective metabolic and pharmacodynamic profile of FLU. In detail, while *S*- and *R*-FLU are almost equipotent in blocking serotonin reuptake, *S*-NFLU is 20-times more potent than *R*-NFLU. CYP2D6 is the major metabolizing isoenzyme of FLU and the main responsible for the formation of *S*-NFLU. Thus, in a scenario where the CYP2D6 functional activity is decreased, there is probably an alternative increase in the *R*-portion and in the less pharmacologically active *R*-NFLU, contributing to decrease the efficacy (97,98). Additionally, it has been documented that CYP2D6 is involved in the cerebral production of dopamine and serotonin by an alternative pathway. In line with these facts, poor antidepressant therapeutic response has been described in CYP2D6 gPMs (68).

The most interesting finding of this second work was the identification of genetic and non-genetic factors related to P-gp as potential biomarkers of the clinical outcomes of FLU. Specifically, the *ABCB1 TTT*-haplotype was favourable to better clinical outcomes with FLU (lower severity of adverse effects and higher likelihood of remission) and the potential of the P-gp to be inhibited by drug-induced phenoconversion was associated to a worse tolerability profile (higher severity and number of adverse effects). Considering that this *T*-allele in the *ABCB1 1236C>T, 2677G>T/A* and *3435C>T* SNPs has been linked to the increasing of the cerebral bioavailability of P-gp substrates, these two last results seem contradictory, but they are not. Recent evidence show that the *T*-allele in these SNPs is a better antidepressant response allele, but it is also responsible for the decreasing of tolerability, since it is associated with lower P-gp expression at the blood-brain barrier, easier access and accumulation of the P-gp substrate antidepressant drug into the brain, increasing the potential of adverse effects (87). In other words, the decrease of the P-gp activity and consequent increase of the FLU cerebral bioavailability appears to be therapeutically advantageous at some extent, because it increases the potential of efficacy. However, at certain point, the decrease of the P-gp activity leads to the decrease of the tolerability of the drug due to brain accumulation. Moreover, it has been described that a decreased P-gp function at the blood–brain barrier may contribute to the pathophysiology of some mood disorders, including depression, due to the accumulation of toxins in the brain (98). In agreement, *ABCB1 TTT-TTT* haplotype has been related with poor psychiatric outcomes, namely violent suicide attempts (173). Thus, these facts explain why the carriers of the *ABCB1 TTT*-haplotype presented better clinical outcomes

with FLU than those with the *TTT-TTT* haplotype, but also why patients with the potential of the P-gp to be inhibited were associated to worse tolerability profile. Such results are of particular scientific relevance, because FLU has been overall considered as a non-P-gp-substrate and, therefore, there are scarce data on this matter (87,98).

Other non-genetic factors that have been described as clinically relevant moderators of the antidepressant clinical outcomes were found as potential biomarkers for FLU in the *GnG-PK/PD-AD* study (225,229). The presence of nervous system co-morbidities was associated with higher severity of adverse effects, while aging and the female gender were associated with higher severity of depression and lower probability of remission. At this level it should be mentioned that gender differences in the antidepressant therapeutic response remains a controversial topic, as consequence of the common gender bias favouring females (228,230,320). Bearing in mind that the *GnG-PK/PD-AD* study also presented a marked overrepresentation of female patients, the conclusions related to gender should be viewed with caution.

Finally, it is important to highlight that the *GnG-PK/PD-AD* study should be viewed as an exploratory work, due to its relatively small sample size and cross-sectional design. As previously discussed, the *GnG-PK/PD-AD* study was based on a cross-sectional design and it presents a limited sample size, particularly for sub-populational analyses. Additionally, the clinical assessments were performed at one single time-point and no pre-treatment and follow-up data were available due to the naturalistic clinical setting of the study. Still, real-world studies are tremendously complex and other non-studied factors, such as genetic factors related to pharmacodynamics, might impact the results. Despite these study limitations, the use of a "real world" sample increases the extrapolation capacity and representativeness of the study findings. Also, the power of the study is increased thanks to the homogeneity in terms of antidepressant treatment (only three antidepressants in study) and to the integrated study approach, involving the assessment of genetic and non-genetic factors.

IV.2 CONCLUSION AND FUTURE PERSPECTIVE

IV.2 CONCLUSION AND FUTURE PERSPECTIVE

The present doctoral work affords a comprehensive pharmacometric evaluation of the clinical impact of genetic polymorphisms of the CYP and P-gp, as well as of non-genetic factors, on the pharmacokinetics and pharmacodynamics of antidepressant drugs, specifically FLU, PAR and VEN. Overall, the objectives defined for this doctoral project were successfully achieved.

Initially, two bioanalytical MESP/HPLC-FLD methods were successfully developed and validated for the quantification of VEN+ODV and FLU+NFLU+PAR, respectively, which supported the therapeutic drug monitoring approach planned in the *GnG-PK/PD-AD* study. These are highly throughput and cost-effective techniques appropriate for therapeutic drug monitoring and for other pharmacokinetic studies and constitute an important stimulus to the implementation of the pharmacokinetic monitoring in the clinic, guiding the therapeutic decisions/interventions. Furthermore, these methods are the reflex of the paradigm shift that the clinical bioanalysis is currently living, towards the use of micro-sampling and miniaturization systems for sample preparation and analysis.

In turn, the clinical phase and the phase of pharmacometric analysis of this doctoral work were also successfully carried out. At this level, the *GnG-PK/PD-AD* study provided a real-world clinical characterization of Portuguese patients with depression and treated with FLU, PAR and VEN. Particularly, it demonstrated that the treatment of depression with these antidepressant drugs is frequently subject to a high inter-individual variability and poor clinical outcomes in the real-world clinical setting. Several genetic and non-genetic factors were identified as potential sources of inter-individual variability of the clinical outcomes with antidepressant drugs. Thus, it was concluded that genetic and non-genetic factors must be studied together. Importantly, the *GnG-PK/PD-AD* study stressed that a high potential of drug-induced phenoconversion is a common scenario in the real-world clinical setting of treatment of depression, as consequence of the common co-morbid and polypharmacy state of the patients. Bearing these findings in mind, the *GnG-PK/PD-AD* study clearly showed that the antidepressant treatments with FLU, PAR and VEN are difficult to guide and manage and that the availability of therapeutic biomarkers is of utmost importance to improve the antidepressant treatments. At this level, potential genetic and non-genetic therapeutic biomarkers were identified for FLU.

Up to now, no clinically relevant therapeutic biomarkers, including pharmacogenetic biomarkers, are available for FLU. Hence, these results are expected to be a relevant contribution for the field of clinical pharmacology and pharmacotherapy of depression. The *GnG-PK/PD-AD* study sample proved to be representative of the real-world clinical setting of the treatment of depression with FLU, PAR and VEN, constituting therefore a dataset with clinical validity for further analyses. Larger studies addressing these issues will be needed in the future to confirm the results described in the present doctoral work.

Moreover, the present doctoral work emphasizes the co-integrated effect of genetic and non-genetic individual factors on the modulation of the real phenotype, as well as the potential superiority of combining several genetic and non-genetic factors as composite phenotypes and biomarkers. In fact, the investigation of composite phenotypes is currently one of the main hot-topics in pharmacogenetics. The future challenge that arises for the pharmacogenetics, including at the level of the CYP isoenzymes, is the redefinition of the classic system of phenotype classification, in order to translate and incorporate genetic and non-genetic-induced phenoconversion interactions into a real phenotype. The model herein proposed for the assessment of the potential of drug-induced phenoconversion (DPI score) opens the door for this new paradigm in parallel with the phenotyping tests.

To finish, the present work offers real-world-based evidence to change the mindset of pharmacogenetics and personalized medicine towards the integrated investigation of genetic and non-genetic factors and genotype-phenotype associations in the treatment of depression with antidepressant drugs, aiming the translation of the pharmacogenetics knowledge into clinical practice. The integrated use of pharmacogenetics and therapeutic drug monitoring confirmed to be a complementary and useful approach to identify potential therapeutic biomarkers and to improve the clinical outcomes of antidepressant drugs.

APPENDICES

APPENDIX A – CLINICAL STUDY PROTOCOL AND INFORMED CONSENT FORM OF THE *GnG-PK/PD-AD* STUDY

PROJETO DE INVESTIGAÇÃO NO ÂMBITO DO DOUTORAMENTO EM CIÊNCIAS FARMACÊUTICAS

Avaliação farmacométrica do impacto clínico de polimorfismos genéticos do citocromo P450 e da glicoproteína-P na farmacocinética e farmacodinamia de antidepressivos: fluoxetina, paroxetina e venlafaxina

Pharmacometric evaluation of the clinical impact of genetic polymorphisms in the cytochrome P450 and P-glycoprotein on the pharmacokinetics and pharmacodynamics of antidepressants: fluoxetine, paroxetine and venlafaxine

Investigador Principal: Paulo Ricardo Machado Magalhães [1,2,3]

Orientador: Prof. Doutor Amílcar Falcão [1]
Co-orientadores: Prof. Doutor Gilberto Alves [1, 2] e
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Dezembro de 2014

1. RESUMO

A patologia depressiva tem revelado um impacto negativo crescente na qualidade de vida das sociedades atuais, representando a principal causa de incapacidade mundial. O elevado nível de insucesso farmacoterapêutico, explicado em parte por fatores genéticos que refletem a variabilidade inter-individual na resposta clínica aos antidepressivos, é um extenso contributo para este facto.

Hoje é reconhecido que os polimorfismos nos genes das isoenzimas do citocromo P450 (CYP), tal como no gene da glicoproteína-P podem interferir com o perfil farmacocinético e com a resposta farmacológica. O estudo destas relações farmacogenéticas com a farmacocinética e a farmacodinamia assume interesse particular na individualização da terapia antidepressiva com fármacos de ampla utilização clínica, nomeadamente a fluoxetina, paroxetina e a venlafaxina. Então, numa tentativa de otimizar o tratamento farmacológico, este projeto pretende caraterizar a farmacocinética populacional dos antidepressivos referidos, tendo por base uma avaliação farmacométrica da influência de co-variáveis genéticas, considerando inevitavelmente as variáveis demográficas e clínicas dos doentes.

Para tal, a colaboração de unidades de saúde primárias, em particular de Centros de Saúde, é imprescindível. A este nível, pretende-se especificamente a colaboração das equipas médicas e das equipas de enfermagem (elementos intervenientes).

Assim, tendo em conta o carácter clínico do estudo, serve o presente documento para apresentar a investigação não só às possíveis unidades de saúde envolvidas e respetivos intervenientes, mas também aos demais órgãos competentes e responsáveis pela avaliação e garantia da conformidade deste com todos os pressupostos éticos e morais que a investigação em humanos implica. Com o intuito de objetivar e uniformizar os procedimentos entre os diferentes centros de estudo, é também providenciado por este meio o protocolo de atuação para cada um dos potenciais elementos intervenientes afetos à unidade de saúde a que a investigação está a ser proposta.

2. ESTADO DA ARTE

As perturbações depressivas constituem um problema *major* de saúde pública, com impacto negativo na qualidade de vida e na morbi-mortalidade. Segundo a *Organização Mundial de Saúde* a depressão é a principal causa de incapacidade em todo o mundo (1). Particularmente preocupante é o risco de mortalidade por suicídio nos doentes com depressão, o qual é 20 vezes superior ao da população em geral (2).

Apesar dos avanços no entendimento fisiopatológico da depressão e da disponibilidade clínica de um vasto arsenal de fármacos antidepressivos, com mecanismos de ação distintos, muitos doentes não respondem à farmacoterapia. Dados recentes indicam que aproximadamente metade dos doentes com depressão não responde à primeira opção farmacoterapêutica e, cerca de um terço, não alcança a remissão clínica dos sintomas após serem testados múltiplos fármacos antidepressivos (3). Embora a depressão resistente ao tratamento possa resultar da combinação de múltiplos fatores (fisiopatológicos, ambientais e genéticos), nos últimos anos um enfoque especial tem sido atribuído à farmacogenética enquanto fator determinante para a variabilidade inter-individual observada na resposta clínica aos fármacos antidepressivos (4-6).

Hoje é reconhecido que os polimorfismos nos genes das isoenzimas do CYP450 podem influenciar consideravelmente a eficácia farmacológica e os efeitos adversos associados. Pois, diferentes variantes genéticas podem apresentar diferenças significativas na atividade enzimática, contribuindo, consequentemente, para a variabilidade inter-individual no metabolismo e no perfil farmacológico de exposição sistémica. Entre as enzimas polimórficas do CYP, as variantes genéticas das isoenzimas CYP2A6, CYP2B6, CYP2D6, CYP2C19 e CYP2C9 têm sido as mais estudadas (7,8). Globalmente, as diferenças funcionais destas variantes genéticas traduzem-se em quatro fenótipos principais: metabolizadores lentos, intermédios, rápidos e ultrarrápidos (8). Paralelamente, também a presença de polimorfismos no gene *ABCB1* da glicoproteína-P pode condicionar diferenças importantes na atividade funcional desta glicoproteína (transportador membranar de efluxo); em consequência pode ocorrer uma elevada variabilidade na biodisposição de fármacos substratos da glicoproteína-P, com eventuais implicações clínicas (9).

Nos últimos anos têm sido realizados diversos estudos para avaliar a relevância de variantes genéticas de isoenzimas do CYP ou da glicoproteína-P na resposta aos fármacos antidepressivos. Porém, tais estudos focaram essencialmente as variantes genéticas associadas a um único gene e conduziram a resultados contraditórios (4-6,10). Portanto, sabendo-se que o metabolismo dos fármacos antidepressivos é mediado principalmente pelas isoenzimas polimórficas CYP2D6, CYP2C19 e CYP2C9, e que muitos desses fármacos são também substratos da glicoproteína-P, incluindo ao nível da barreira hematoencefálica, é importante avaliar de forma integrada as implicações da interação de polimorfismos genéticos múltiplos na farmacocinética. Assim, face à variabilidade inter-individual marcada na resposta clínica aos fármacos antidepressivos, espera-se que a identificação de relações entre os polimorfismos genéticos, a farmacocinética e a resposta farmacológica (eficácia terapêutica e perfil de reações adversas) constituirá um dos aspetos mais prometedores para a individualização da farmacoterapia antidepressiva. Neste sentido, afigura-se indispensável a realização de estudos clínicos mais robustos, capazes de permitirem um entendimento mais completo da influência da farmacogenética na farmacocinética e na farmacodinamia de fármacos antidepressivos com ampla utilização clínica, entre eles a fluoxetina, a paroxetina e a venlafaxina.

3. OBJETIVOS

Pretende-se com o trabalho de investigação proposto proceder a uma extensa avaliação farmacométrica da influência de polimorfirmos genéticos de isoenzimas do CYP e da glicoproteína-P na farmacocinética de fármacos antidepressivos utilizados largamente na prática clínica (fluoxetina, paroxetina e venlafaxina), e para os quais se reconhece a existência de variabilidade inter-individual considerável na sua eficácia e tolerabilidade. Efetivamente, o entendimento das implicações de polimorfismos genéticos na resposta farmacológica constitui um dos aspetos mais prometedores no caminho a percorrer em direção à medicina personalizada. É hoje aceite que a variabilidade inter-individual verificada na resposta clínica aos fármacos antidepressivos possa ser determinada, pelo menos em parte, pela expressão de diferentes variantes genéticas de enzimas metabolizadoras e/ou transportadores envolvidos nos processos cinéticos desses fármacos. Na verdade, diferenças marcadas nos perfis de concentrações

séricas têm sido observadas após a administração de doses padrão de fármacos antidepressivos em indivíduos com o fenótipo metabolizador lento *versus* ultrarrápido, condicionando, respetivamente, um risco acrescido para reações adversas *versus* ineficácia clínica. Atendendo a que os esforços recentes dirigidos ao desenvolvimento de novos fármacos antidepressivos têm sido relativamente mal-sucedidos, a farmacogenética clínica surge como uma oportunidade relevante na individualização da farmacoterapia antidepressiva. De facto, as correlações genótipo-fenótipo fornecem a base para predizer o fenótipo individual na sequência de testes genéticos, permitindo assim otimizar os tratamentos farmacológicos.

Portanto, numa tentativa de contribuir para o estabelecimento de correlações genótipo-fenótipo clinicamente robustas, pretende-se com este projeto caraterizar a farmacocinética populacional da fluoxetina, paroxetina e venlafaxina em doentes, considerando nesta análise farmacométrica a influência independente e integrada de co-variáveis genéticas, sem esquecer, inevitavelmente, os dados demográficos e clínicos (com ênfase na co-medicação) dos doentes. Estes resultados permitir-nos-ão tirar ilações sobre a relação farmacogenética/farmacocinética/ farmacodinâmica e inferir acerca do interesse clínico da genotipagem dos doentes para a maximização do binómio segurança/eficácia dos tratamentos com os fármacos propostos. Para tal, o projeto de investigação aqui exposto é subdividido em tarefas e objetivos intermédios que são descritos detalhadamente a seguir.

4. DESCRIÇÃO DETALHADA

TAREFA 1. Desenvolvimento e validação de técnicas analíticas de cromatografia líquida para a determinação quantitativa da fluoxetina, paroxetina, venlafaxina e seus metabolitos em plasma humano. As técnicas serão desenvolvidas e validadas de acordo com as orientações preconizadas e internacionalmente aceites para a validação de métodos bioanalíticos [11,12]. Este facto assume extrema importância, uma vez que a realização de estudos farmacocinéticos fidedignos é sustentada pela disponibilidade de técnicas analíticas quantitativas convenientemente validadas.

TAREFA 2. Recrutamento prospetivo de doentes adultos com diagnóstico de depressão e tratados em regime de ambulatório há **pelo menos dois meses** com um dos três fármacos, fluoxetina, paroxetina e venlafaxina, como **principal opção terapêutica antidepressiva**. Os indivíduos que apresentem algum dos critérios seguintes (critérios de exclusão) não serão incluídos no estudo:

- **Indivíduos incapazes de darem o seu consentimento esclarecido (ex. atraso mental, demências, etc);**
- **Indivíduos menores;**
- **Indivíduos que não querem participar no estudo e/ou que não assinem o consentimento informado.**

Particularmente, é a este nível que se pretende e se solicita a colaboração de Unidades de Saúde primárias abrangidas pela *Administração Regional de Saúde do Centro (ARSC)* no sentido de integrarem o estudo como pontos de recrutamento de participantes. Pelas suas caraterísticas e objetivos, bem como pelos seus requerimentos protocolares, os potenciais intervenientes serão elementos da equipa médica e da equipa de enfermagem de cada Unidade de Saúde e que aceitem colaborar com a investigação aqui proposta.

Em termos de duração do processo de recrutamento multicêntrico, perspetiva-se um período mínimo de ano e meio, após parecer favorável e aprovação por parte da *Comissão de Ética da ARSC*, com o objetivo de constituir uma amostra final de pelo menos 100-150 doentes por antidepressivo. Cada participante será abordado e sujeito ao protocolo do estudo *(Anexo 1.1)* num único ponto do tempo, correspondendo, portanto, a um estudo de caris transversal. O recrutamento/sensibilização dos participantes será levado a cabo pelos clínicos que aceitem colaborar através das suas consultas, considerando os critérios de inclusão e de exclusão acima propostos.

Aos participantes ser-lhe-á aplicado um questionário devidamente estruturado e adaptado, o qual se destina a recolher informação clínica relevante, bem como a avaliar a severidade da depressão e a resposta ao tratamento antidepressivo (eficácia e efeitos adversos) *(Anexo 1.5)*. Este é constituído pela *Ficha do Participante* e pela *Escala de Avaliação da Depressão de Hamilton, versão 17 itens (HAM-D_{17})* [13], os quais deverão ser preenchidos pelo clínico mediante entrevista ao doente, assim como pela *Checklist de*

efeitos adversos aos antidepressivos (ASEC) [14] que é dirigida para o preenchimento do participante. Posteriormente, poderá o investigador solicitar ao médico assistente do participante, e de acordo com este, informações sobre o processo clínico. Importa também referir que os elementos colaborantes a este nível serão considerados como co-autores dos resultados provenientes da investigação em questão e, portanto, tidos em conta em futuras publicações que daí advenham.

Após a intervenção do médico/investigador, os doentes incluídos no estudo serão submetidos à colheita de uma amostra de sangue (cerca de 5-10 mL) por parte de um elemento da equipa de enfermagem. Neste âmbito é importante o registo da hora da colheita na *Ficha do Participante (Anexo 1.5.1)*. As amostras de sangue obtidas serão armazenadas de forma apropriada para posterior análise farmacocinética (concentração de fármaco e/ou metabolito ativo *versus* tempo) e farmacogenética (genotipagem de isoenzimas CYP e glicoproteína-P) *(tarefa 3,4 e 5)*.

Todo este processo será articulado entre o investigador e os elementos das equipas médicas e das equipas de enfermagem, tendo por base o protocolo detalhado que é apresentado no *anexo 1.1*, o qual, por conveniência, também integrará cada *Dossier* do Participante (*Anexo 1)*. Importa ainda ressalvar que todo o material necessário à recolha, armazenamento/acondicionamento das amostras será cedido pelo investigador.

TAREFA 3. Determinação quantitativa das concentrações plasmáticas de fluoxetina, paroxetina, venlafaxina e seus metabolitos ativos, nas amostras obtidas a partir dos doentes incluídos no estudo, mediante a aplicação das técnicas analíticas previamente desenvolvidas na tarefa 1. No final desta etapa, a análise dos pares de dados concentração (fármaco/metabolito) versus tempo normalizados pela dose administrada de fármaco antidepressivo poderá permitir a classificação do fenótipo individual dos doentes.

TAREFA 4. Genotipagem dos genes das isoenzimas polimórficas CYP2D6, CYP2C19 e CYP2C9, e da glicoproteína-P (*MDR1 ou ABCB1*) a partir das amostras de sangue periférico obtidas dos doentes. O objetivo desta tarefa é a determinação para cada doente das variantes genéticas das isoenzimas do CYP ou da glicoproteína-P relevantes para o metabolismo e biodisposição dos fármacos antidepressivos selecionados (fluoxetina, paroxetina e venlafaxina).

TAREFA 5. Análise e caraterização farmacocinética/farmacodinâmica dos fármacos antidepressivos propostos (fluoxetina, paroxetina e venlafaxina), conjugando a informação da genotipagem dos genes das isoenzimas CYP2D6, CYP2C9, CYP2C19 e *MDR1* ou *ABCB1,* os pares de dados concentração (fármaco/metabolito) *versus* tempo obtidos, e os fatores demográficos, clínicos e terapêuticos dos doentes (co-medicação e resposta clínica).

5. CONSIDERAÇÕES ÉTICAS

Tendo por base a existência de contacto direto com os doentes e a importância de salvaguardar os seus direitos e vontades, importa referir que este estudo será desenvolvido de acordo com os princípios de *Boas Práticas Clínicas*, com a *Declaração de Helsínquia* e suas subsequentes revisões, com a *Declaração Universal da UNESCO sobre o Genoma Humano e os Direitos Humanos* e com a *Lei Nacional nº67/98 de 26 de Outubro – Lei de Proteção de Dados.* Em consonância com esta última, a recolha e o processamento de dados pessoais carece de notificação prévia à *Comissão Nacional de Proteção de Dados (CNPD)*, pelo que este processo será devidamente comunicado antes do início do recrutamento clínico.

Todos os participantes serão extensivamente informados sobre todos os aspetos do estudo por meio oral e escrito, sendo requerido a assinatura de um consentimento informado *(Anexo 1.3 e 1.4)*. De modo a assegurar o anonimato dos participantes, todos os seus dados serão sempre abordados com carácter ético e sigiloso, mediante o uso de códigos de correspondência. Estes serão armazenados pelo investigador principal em parceria com os centros de investigação associados. É também de salientar que os resultados da investigação podem vir a ser apresentados em encontros, conferências, e pósteres científicos. O anonimato do participante será sempre garantido.

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ANEXO 1

DOSSIER DO PARTICIPANTE DO ESTUDO:

Avaliação farmacométrica do impacto clínico de polimorfismos genéticos do citocromo P450 e da glicoproteína-P na farmacocinética e farmacodinamia de antidepressivos: fluoxetina, paroxetina e venlafaxine

Investigador Principal: Paulo Ricardo Machado Magalhães

CONTEÚDO:

1.1 Protocolo detalhado de atuação para os elementos intervenientes no estudo com checklist;

1.2 Ficha de Informação ao Participante;

1.3 e 1.4 Consentimento Informado *(em duplicado, um para o participante e outro para o investigador)*;

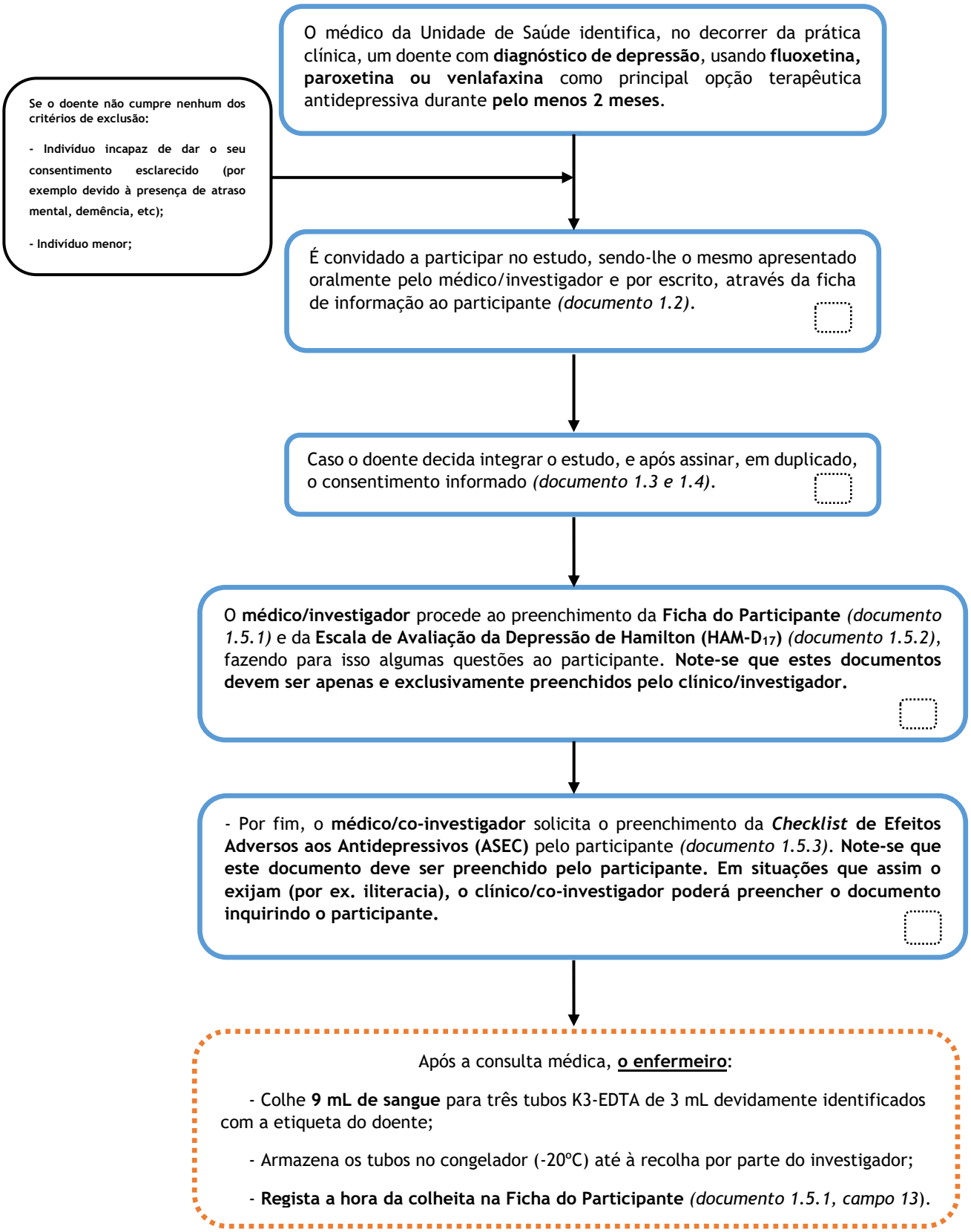
1.5 Questionário ao participante

1.5.1 Ficha do Participante;

1.5.2 Escala de avaliação da depressão de Hamilton (HAM-D_{17});

1.5.3 *Checklist* de efeitos adversos aos antidepressivos (ASEC).

1.1 PROTOCOLO DE ATUAÇÃO PARA OS ELEMENTOS INTERVENIENTES NO ESTUDO COM *CHECKLIST*



1.2 FICHA DE INFORMAÇÃO AO PARTICIPANTE *(documento para o participante)*

Título: *Avaliação farmacométrica do impacto clínico de polimorfismos genéticos do citocromo P450 e da glicoproteína-P na farmacocinética e farmacodinamia de antidepressivos: fluoxetina, paroxetina e venlafaxina*

Investigador Principal: Paulo Ricardo Machado Magalhães

O seu médico sugeriu a sua integração no estudo acima mencionado uma vez que o(a) senhor(a) tem uma doença que requer o tratamento com um dos seguintes medicamentos antidepressivos: fluoxetina, paroxetina ou venlafaxina.

Este documento pretende explicar-lhe o assunto e o objetivo do estudo, ajudando-o a tomar a decisão. Antes de decidir se aceita ou não integrar o estudo, é importante que compreenda o motivo pelo qual esta investigação será realizada, assim como o que será envolvido.

QUAL É A PROPOSTA DO PRESENTE ESTUDO?

O estudo que lhe está a ser proposto é intitulado: "Avaliação farmacométrica do impacto clínico de polimorfismos genéticos do citocromo P450 e da glicoproteína-P na farmacocinética e farmacodinamia de antidepressivos: fluoxetina, paroxetina e venlafaxina*".*

A farmacocinética é o estudo dos processos a que o corpo sujeita o medicamento; enquanto a farmacodinamia é o estudo do que o medicamento faz ao corpo; por sua vez a farmacogenética estuda a variabilidade na resposta farmacológica devido a fatores hereditários. Estes fatores podem levar a que as pessoas respondam de formas diferentes aos medicamentos.

Então, este estudo pretende avaliar para a fluoxetina, paroxetina e venlafaxina as relações entre estes três campos, com o objetivo de melhorar os resultados com estes medicamentos, alcançando maior eficácia e menos efeitos adversos.

A sua participação neste estudo envolve a doação de uma amostra de sangue, que será colhida por um elemento da equipa de enfermagem. Adicionalmente ser-lhe-ão submetidas algumas questões pelo médico de forma a preencher um questionário que pretende recolher informação sociodemográfica e clínica. No fim da intervenção, após a colheita da amostra de sangue pelo enfermeiro(a), ser-lhe-á solicitado que preencha um formulário destinado a recolher informação sobre eventuais efeitos adversos relacionados

com o antidepressivo que toma. O seu processo clínico poderá também ser revisto. Este estudo não envolve qualquer outro procedimento.

O QUE EU TENHO DE FAZER DURANTE O ESTUDO?

O estudo não exige que seja tido em conta nenhum comportamento específico, nem o perturbará a qualquer nível, uma vez que será realizado no âmbito de uma consulta de rotina. Apenas será necessário que responda com sinceridade às questões que lhe serão colocadas, quer diretamente pelo médico quer pelo questionário que lhe será proposto.

QUAIS SÃO OS BENEFÍCIOS E OS RISCOS DE PARTICIPAR NO ESTUDO?

O participante não terá qualquer benefício direto através deste estudo. Contudo, a sua participação nesta investigação pode ajudar a melhorar o conhecimento científico da sua doença e a melhorar o seu tratamento.

Os riscos e os desconfortos físicos resultantes da participação no estudo serão aqueles associados à colheita de sangue, entre eles: dor ligeira, irritação, edema e muito raramente infeção.

QUAIS SERÃO OS PROCEDIMENTOS RELATIVOS À CONFIDENCIALIDADE DA MINHA IDENTIDADE?

Ao assinar o Consentimento Informado estará a autorizar o uso da sua amostra biológica e de dados clínicos para investigação. A sua amostra e os seus dados serão apenas utilizados para o fim que é aqui proposto.

Todos os seus dados serão sempre abordados com carácter ético e sigiloso, mediante o uso de códigos de correspondência, que asseguram o seu anonimato. Estes serão armazenados pelo investigador principal em parceria com os centros de investigação associados.

Importa ainda referir que os resultados do estudo podem vir a ser apresentados em encontros, conferências, e pósteres científicos. O seu anonimato será sempre garantido.

RECEBEREI ALGUMA COMPENSAÇÃO ECONÓMICA?

Não receberá qualquer compensação monetária pela sua participação neste estudo ou pelos resultados obtidos.

O QUE ACONTECERÁ SE EU NÃO PRETENDER INTEGRAR O ESTUDO, OU SE PRETENDER DESISTIR APÓS ASSINAR O CONSENTIMENTO INFORMADO?

A participação neste estudo é voluntária. É da sua completa autonomia decidir em participar ou não. Se decidir não participar, isto não afetará os cuidados de saúde que lhe são prestados, seja agora ou no futuro.

Seja qual for a decisão, esta não irá interferir com a relação entre si e os diferentes profissionais de saúde.

Se desejar abandonar o estudo, mesmo após este ter iniciado, poderá fazê-lo a qualquer momento, sem dar qualquer justificação. Neste caso, a sua amostra e os seus dados serão destruídos.

O QUE DEVO FAZER SE TIVER ALGUMA QUESTÃO?

Se surgir alguma questão ou necessitar de algum esclarecimento agora, ou a qualquer momento durante o estudo, por favor contacte:

Nome do Investigador Principal: Paulo Ricardo Machado Magalhães

1.3 CONSENTIMENTO INFORMADO *(versão para o participante)*

Título: *Avaliação farmacométrica do impacto clínico de polimorfismos genéticos do citocromo P450 e da glicoproteína-P na farmacocinética e farmacodinamia de antidepressivos: fluoxetina, paroxetina e venlafaxina*

Eu, *(Nome completo)* _____

_____

_____declaro:

- que li e compreendi a ficha de informação ao participante;
- que recebi informação suficiente relativa a este estudo;
- que as minhas questões foram satisfatoriamente esclarecidas;
- que tive tempo suficiente para ponderar a minha decisão;
- que compreendi o carácter totalmente voluntário da minha participação;

De acordo com as considerações apresentadas na ficha de informação ao participante, **eu aceito participar voluntariamente no presente estudo**.

_____ de 20_____

Assinatura do Participante: Assinatura do médico:

_____ _____

Assinatura do Investigador

_____

(Paulo Magalhães)

1.4 CONSENTIMENTO INFORMADO *(versão para o investigador)*

Título: *Avaliação farmacométrica do impacto clínico de polimorfismos genéticos do citocromo P450 e da glicoproteína-P na farmacocinética e farmacodinamia de antidepressivos: fluoxetina, paroxetina e venlafaxina*

Eu, *(Nome completo)* _____

_____

_____declaro:

- que li e compreendi a ficha de informação ao participante;
- que recebi informação suficiente relativa a este estudo;
- que as minhas questões foram satisfatoriamente esclarecidas;
- que tive tempo suficiente para ponderar a minha decisão;
- que compreendi o carácter totalmente voluntário da minha participação;

De acordo com as considerações apresentadas na ficha de informação ao participante, **eu aceito participar voluntariamente no presente estudo**.

_____ de 20_____

Assinatura do Participante: Assinatura do médico:

_____ _____

Assinatura do Investigador

_____

(Paulo Magalhães)

1.5 QUESTIONÁRIO AO PARTICIPANTE

1.5.1 FICHA DO PARTICIPANTE

O presente formulário pretende recolher informação sociodemográfica e clínica do participante, assim como outros dados relevantes para a investigação. **Este deve ser preenchido pelo médico/co-investigador interveniente** no estudo através de questões colocadas ao doente e/ou consulta do seu historial clínico.

1. Idade: _____

2. Sexo: a) Masculino b) Feminino

3. Grupo racial:

a) Caucasiano b) Asiático c) Africano d) Americano nativo

4. Qual a duração aproximada do presente episódio depressivo?

a) 2 a 6 meses b) 6 meses a 1ano c) 1 a 2 anos d) mais de 2 anos

5. Qual é o medicamento antidepressivo usado atualmente?

a) Fluoxetina b) Paroxetina c) Venlafaxina

6. Qual é o regime posológico usado?

_____ mg/_____ (dose/intervalo de tempo)

7. Há quanto tempo toma este antidepressivo para o presente episódio depressivo?

a) 2 a 6 meses b) 6 meses a 1 ano c) 1 a 2 anos d) mais de 2 anos

8. Já utilizou anteriormente outro tratamento farmacológico para o presente episódio depressivo?

a) Sim. Qual? _____ b) Não

9. Se sim, qual foi o motivo que levou à descontinuação desse tratamento farmacológico e à mudança para o medicamento antidepressivo que toma atualmente?

a) Efeitos adversos b) Ausência de melhoria clínica c) Interações farmacológicas

d) Outro. Qual? _____

10. Co-morbilidades:_____

_____

11. Co-medicação:_____

_____

12. Data e hora da última toma (aproximada): _____/_____/_____ ; _____ h _____ min

13. Data e hora da colheita da amostra: _____/_____/_____; _____ h _____ min

1.5.2 ESCALA DE AVALIAÇÃO DA DEPRESSÃO DE HAMILTON (HAM-D_{17})

O presente formulário pretende avaliar a severidade do episódio depressivo do participante e a sua resposta ao tratamento. **Este também deve ser preenchido pelo médico/co-investigador interveniente** no estudo através de questões colocadas ao doente. Para tal, a maioria dos itens da escala (do 1 ao 17) apresentam questões guia que permitem obter a informação adequada para preencher cada um dos campos. Por favor, em cada item **a negrito** selecione a opção (de 0 a 4 ou 0 a 2) que melhor traduz o estado do participante **na última semana**.

Gostaria de lhe fazer algumas perguntas sobre a última semana.

1. Humor depressivo (tristeza, desesperança, desamparo, inutilidade)

Questão 1: Como tem estado o seu humor na última semana? O Senhor(a) tem-se sentido em baixo ou deprimido(a)? Triste? Sem esperança? Tem chorado?

0- Ausente;

1- Sentimentos relatados somente quando perguntados;

2- Sentimentos relatados espontaneamente, com palavras;

3- Comunica os sentimentos não com palavras, mas com a expressão facial, postura, voz e tendência de choro;

4- Sentimentos deduzidos da comunicação verbal e não-verbal do doente.

2. Sentimentos de culpa:

Questão 2: O senhor(a) tem-se sentido especialmente autocrítico nesta última semana, sentindo que fez coisas erradas ou que dececionou outras pessoas? Tem-se sentido culpado? Tem pensado que, de alguma forma, é responsável pela sua depressão e que está a ser castigado ao estar doente?

0- Ausente;

1- Autorrecriminação; sente que dececionou os outros;

2- Ideias de culpa ou ruminação sobre erros passados ou más ações;

3- A doença atual é uma punição;

4- Ouve vozes de acusação ou denúncia e/ou tem alucinações visuais ameaçadoras.

3. Suicídio:

Questão 3: Nesta última semana, o Senhor(a) teve pensamentos de que não vale a pena viver ou que estaria melhor morto? Ou pensamentos de se magoar ou até de se matar?

0- Ausente;

1- Sente que a vida não vale a pena;

2- Deseja estar morto ou pensa na probabilidade da sua própria morte;

3- Ideias ou atitudes suicidas;

4- Tentativas de suicídio.

4. Insónia inicial:

Questão 4: Como tem sido o seu sono na última semana? O Senhor(a) teve alguma dificuldade em iniciar o sono?

0- Sem dificuldades para iniciar o sono;

1- Queixa-se de dificuldade ocasional para iniciar o sono, ou seja, mais do que meia hora;

2- Queixa-se de dificuldade para iniciar o sono todas as noites.

5. Insónia intermédia:

Questão 5: Durante esta última semana, sente que o seu sono tem sido agitado ou perturbado? Tem acordado a meio da noite?

0- Sem dificuldade;

1- Queixa de agitação e perturbação durante a noite;

2- Acorda durante a noite (qualquer saída da cama, exceto por motivos de necessidade fisiológica).

6. Insónia tardia:

Questão 6: Como tem sido o seu sono na última fase da noite? (ler as opções)

0- Sem dificuldade;

1- Acorda durante a madrugada, mas volta a dormir;

2- Não consegue voltar a dormir quando se levanta da cama durante a noite.

7. Trabalho e atividades:

Questão 7: Como tem passado o seu tempo na última semana? Tem trabalhado? Tem feito outras atividades que para além do trabalho? Tem tido vontade de fazer essas atividades?

0- Sem dificuldades;

1- Pensamentos e sentimentos de incapacidade, fadiga ou fraqueza, relacionados com as atividades, trabalho ou passatempos;

2- Perda de interesse em atividades, passatempos ou trabalho, quer relatado diretamente pelo doente, quer indiretamente por desatenção, indecisão ou hesitação (sente que precisa de se esforçar para o trabalho e para outras atividades);

3- Diminuição do tempo gasto em atividades ou queda de produtividade.

4- Parou de trabalhar devido à doença.

8. Retardo (lentificação do pensamento e da fala, dificuldade de concentração, diminuição da atividade motora) *(avaliação com base na observação):*

0- Pensamentos e fala normais;

1- Lentificação discreta na entrevista;

2- Lentificação óbvia durante a entrevista;

3- Entrevista difícil;

4- Imobilidade completa.

9. Agitação *(avaliação com base na observação)***:**

0- Nenhuma;

1- Inquietação;

2- Mexe as mãos, cabelos etc.;

3- Movimenta-se bastante, não consegue permanecer sentado durante a entrevista;

4- Retorce as mãos, rói as unhas, puxa os cabelos, morde os lábios.

10. Ansiedade - psíquica:

Questão 10: Tem-se sentido especialmente tenso ou irritado nesta última semana? Tem-se preocupado com coisas pouco importantes com as quais normalmente não se preocuparia?

0- Sem dificuldade;

1- Tensão e irritabilidade subjetiva;

2- Preocupa-se com trivialidades;

3- Atitude apreensiva que é aparente no rosto ou na fala;

4- O doente expressa medo sem ser perguntado.

11. Ansiedade - somática:

Questão 11: Na última semana, o senhor(a) sofreu de alguns dos seguintes sintomas físicos associados a ansiedade? (Leia a lista)

Boca seca, flatulências, indigestão, diarreia, cólicas, eructações (libertação de ar pela boca), palpitações, cefaleias, hiperventilação, suspiros, frequência em urinar, sudorese (transpiração excessiva).

0- Ausente;

1- Leve;

2- Moderado;

3- Severo;

4- Incapacitante.

12. Sintomas somáticos – gastrointestinais

Questão 12: Como tem estado o seu apetite nesta última semana? (ler opções)

0- Normal;

1- Perda de apetite, mas come sem necessidade de insistência;

2- Dificuldade para comer se não insistirem.

13. Sintomas somáticos - gerais:

Questão 13: Nesta última semana como se tem sentido em termos energéticos e de cansaço?

0- Nenhum;

1- Peso em membros, costas ou cabeça; dor nas costas, na cabeça ou nos músculos. Perda de energia e fadiga;

2- Qualquer outro sintoma bem caracterizado e claro.

14. Sintomas Genitais:

14. Tem sentido perda de interesse sexual e/ou (se aplicável) distúrbios menstruais?

0- Ausentes;

1- Leves ou infrequentes;

2- Severos.

15. Hipocondria:

15. Na última semana, tem-se preocupado muito com a sua saúde? Tem-se queixado muito por sintomas físicos?

0- Ausente;

1- Auto-observação aumentada;

2- Preocupação com a saúde;

3- Queixas frequentes, pedidos de ajuda etc.;

4- Delírios hipocondríacos.

16.Perda de Peso:

16. O senhor(a) perdeu peso desde que a depressão teve inicio?

0- Sem perda de peso;

1- Leve ou provável perda de peso associada com a depressão;

2- Definitiva perda de peso causada pela depressão.

17. Consciência *(avaliar com base na observação):*

0- Reconhece estar deprimido e doente;

1- Reconhece estar, mas atribui a causa à má alimentação, ao clima, ao excesso de trabalho, a um vírus, à necessidade de descanso etc.;

2- Nega estar doente.

Adaptado de *Hamilton Rating Scale for depression, Hamilton, M. A rating scale for depression. Journal of Neurology, Neurosurgery, and Psychiatry. 1960, 23, 56-62.*

1.5.3 *CHECKLIST* DE EFEITOS ADVERSOS AOS ANTIDEPRESSIVOS (ASEC)

O presente formulário pretende avaliar a presença de efeitos adversos relacionados com a medicação antidepressiva. **Este destina-se ao preenchimento por parte do participante**. Por favor, classifique a seguinte lista de sintomas numa escala de 0 a 3 tendo por base a sua presença ou não e o seu grau de severidade **(0= ausente; 1= leve; 2= moderado; 3= severo).** Adicionalmente indique se é provável que o sintoma seja um efeito adverso da sua medicação antidepressiva **(sim ou não)**. Escreva um comentário que forneça informação importante quando o sintoma não é um efeito adverso.

	Sintoma	**Escala (0= ausente; 1= leve; 2= moderado; 3= severo)**				**Relacionado com o antidepressivo?**		**Comentário**
1	Boca seca	0	1	2	3	Sim	Não	
2	Sonolência	0	1	2	3	Sim	Não	
3	Insónia (dificuldade em dormir)	0	1	2	3	Sim	Não	
4	Visão turva	0	1	2	3	Sim	Não	
5	Dor de cabeça	0	1	2	3	Sim	Não	
6	Obstipação	0	1	2	3	Sim	Não	
7	Diarreia	0	1	2	3	Sim	Não	
8	Aumento do apetite	0	1	2	3	Sim	Não	
9	Diminuição do apetite	0	1	2	3	Sim	Não	
10	Náuseas ou vómitos	0	1	2	3	Sim	Não	
	1=náuseas ligeiras, 2= náuseas moderadas; 3= náuseas com vómito							
11	Problemas em urinar	0	1	2	3	Sim	Não	
12	Problemas com a função sexual	0	1	2	3	Sim	Não	
13	Palpitações	0	1	2	3	Sim	Não	

	Sintoma	Escala (0= ausente; 1= leve; 2= moderado; 3= severo)				Relacionado com o antidepressivo?		Comentário
14	Tonturas quando está de pé	0	1	2	3	Sim	Não	
15	Sensação de que as coisas estão a girar à sua volta	0	1	2	3	Sim	Não	
16	Sudorese	0	1	2	3	Sim	Não	
17	Aumento da temperatura corporal	0	1	2	3	Sim	Não	
18	Tremores	0	1	2	3	Sim	Não	
19	Desorientação	0	1	2	3	Sim	Não	
20	Fadiga/bocejar	0	1	2	3	Sim	Não	
21	Aumento de peso	0	1	2	3	Sim	Não	

Responda às seguintes questões:

1 – Que outros sintomas tem tido desde que iniciou a sua medicação antidepressiva e que pensa poderem ser efeitos adversos da medicação?

_____

2- Tem feito algum tratamento para os potenciais efeitos adversos? _____

3- Teve algum efeito adverso que o levou à descontinuação da medicação antidepressiva?

_____

Adaptado de Antidepressant Side-Effect Checklist (ASEC), Uher, R. et al. Adverse reactions to antidepressants. The British Journal of Psychiatry. 2009, 195(3), 202–10.

APPENDIX B - AUTHORISATION OF THE ETHICS COMMITTEE AND PORTUGUESE DATA PROTECTION AUTHORITY

Ministério da Saúde

ARSC ADMINISTRAÇÃO REGIONAL DE SAÚDE DO CENTRO, I.P.

Exmo. Senhor
Dr. Paulo Magalhães

paulo.r.magalhaes1@gmail.com

001794 '15 01-26 15:31

Sua referência	Data	Nossa referência	Data

ASSUNTO: "Avaliação farmacométrica do impacto clínico de polimorfismos genéticos do citocromo P450 e da glicoproteína-P na farmacocinética e farmacodinamia de antidepressivos: fluoxetina, paroxetina e venlafaxina".

Serve o presente para informar V. Exª., que o Conselho Diretivo da ARS Centro, I.P., deliberou homologar o parecer emitido pela Comissão de Ética para a Saúde (CES), que se anexa.

Com os melhores cumprimentos

O Presidente do Conselho Diretivo da ARSC, I.P.

(Dr. José Manuel Azenha Tereso)

Na resposta indicar a nossa referência. Em caso de se tratar só de um assunto.

AL

Alameda Júlio Henriques
Apartado 1087 | 3001-553 Coimbra

Telefone: 239 796 800
Fax: 239 796 861

secretariado.ca@arscentro.min-saude.pt
www.arscentro.min-saude.pt

GOVERNO DE PORTUGAL
MINISTÉRIO DA SAÚDE

ARSC ADMINISTRAÇÃO REGIONAL DE SAÚDE DO CENTRO, I.P.

COMISSÃO DE ÉTICA PARA A SAÚDE

PARECER FINAL: Favorável.	DESPACHO: Homologo-se o parecer final da Comissão de Ética para a Saúde 22.1.2015 Conselho Directivo da A.R.S. do Centro I.P. Dr. José Manuel Azenha Tereso Presidente, Dr. Luís Manuel Simão Mendes Cabral Vogal, Drª Maria Augusta Mota Vogal,

ASSUNTO: **Estudo** 58/2014 - "Avaliação farmacométrica do impacto clínico de polimorfismos genéticos do citocromo P450 e da glicoproteína-P na farmacocinética e farmacodinamia de antidepressivos: fluoxetina, paroxetina e venlafaxina"

Autores: Paulo Magalhães (aluno de doutoramento sob a orientação dos Professores Amílcar Falcão, Gilberto Alves Adrián Llerena)

Os autores pretendem estudar a influência de polimorfismos genéticos a nível de CYPs e glicoproteína-P na farmacocinética de três antidepressores: fluoxetina, paroxetina e venlafaxina. Para isso, há uma consulta com o seu médico e são aplicadas diversas escalas (para a depressão e para as reacções adversas). Entretanto é colhida uma amostra de sangue para medir a concentração do fármaco e para fazer a genotipagem. Depois realizar-se-ão diversas correlações farmacocinéticas-farmacodinâmicas.
Há autorização da CNPD.
O consentimento informado está em "boa e devida forma", sendo assinado também pelo médico.
Os princípios éticas estão salvaguardados.

O relator,

Carlos A. Fontes Ribeiro

14 de janeiro de 2015

COMISSÃO NACIONAL
DE PROTECÇÃO DE DADOS

AUTORIZAÇÃO N.º 9426 /2014

Paulo Ricardo Machado Magalhães, após ter sido notificado da Autorização n.º 8844/2014, concedida no âmbito do processo n.º 8384/2014, solicitou a retificação da mesma por não explicitar que a população do estudo é composta de 100 a 150 doentes por cada um dos antidepressivos, das unidades de saúde abrangidas pela Administração Regional de Saúde do Centro.

Assim, com fundamento em erro, nos termos do artigo 141.º do Código do Procedimento Administrativo delibera-se revogar a Autorização n.º 8844/2014 e conceder nova autorização, que segue:

I. Pedido

Paulo Ricardo Machado Magalhães notificou à Comissão Nacional de Proteção de Dados (CNPD) um tratamento de dados pessoais, no âmbito da sua Tese de Doutoramento em Ciências Farmacêuticas, com a finalidade de elaborar um estudo clínico intitulado "Avaliação Farmacométrica do Impacto Clínico de Polimorfismos Genéticos do Citocromo P450 e da Glicoproteína-P na Farmacodinamia de Antidepressivos: Fluoxetina, Paroxetina e Venlafaxina".

A amostra populacional do estudo será composta por 100 a 150 doentes adultos com diagnóstico de depressão e tratados em regime de ambulatório há pelo menos dois meses, por cada um dos três fármacos objeto da investigação – Fluoxetina, Paroxetina e Venlafaxina – nas unidades de saúde abrangidas pela Administração Regional de Saúde do Centro.

Rua de São Bento, 148-3º • 1200-821 LISBOA
Tel: 213 928 400 Fax: 213 976 832
www.cnpd.pt

21 393 00 39
LINHA PRIVACIDADE
Dias úteis das 10 às 13 h

COMISSÃO NACIONAL
DE PROTECÇÃO DE DADOS

A participação no estudo consistirá na resposta a um questionário destinado a recolher a informação clínica relevante, bem como a avaliar a severidade da depressão e a resposta ao tratamento antidepressivo. O preenchimento será levado a cabo pelo médico assistente mediante entrevista ao doente no âmbito da consulta e após recolha do seu consentimento informado.

O médico assistente recolherá ainda uma amostra de sangue na qual é também aposto o código do doente, para análise farmacocinética (concentração de fármaco e/ou metabolito ativo *versus* tempo) e farmacogenética (genotipagem de isoenzimas CYP e Glicoproteína-P).

Os dados serão recolhidos num "caderno de recolha de dados" em formato electrónico, no qual não há identificação nominal do titular, sendo aposto um código de doente. A chave desta codificação só pode ser conhecida da equipa de investigação.

Os destinatários são ainda informados sobre a natureza facultativa da sua participação e garantida confidencialidade no tratamento, caso decidam participar, recolhendo o médico assistente o seu consentimento informado para o efeito.

II. Análise

A CNPD já se pronunciou na sua Deliberação n.º 227/2007 sobre o enquadramento legal, os fundamentos de legitimidade, os princípios orientadores para o correto cumprimento da Lei n.º 67/98, de 26 de outubro (Lei de Proteção de Dados – LPD), bem como as condições gerais aplicáveis ao tratamento de dados pessoais para a finalidade de estudos de investigação na área da saúde.
Assim, enquadrando-se o caso em apreço no âmbito tipificado pela referida Deliberação, porque referentes à saúde e à vida privada, os dados recolhidos pela requerente têm a natureza de sensíveis, razão pela qual o respetivo tratamento só pode basear-se no consentimento expresso, esclarecido e livre dos titulares dos dados, ou dos seus representantes legais, nos termos do disposto no n.º 2 do artigo 7.º da LPD.

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Entende-se por consentimento qualquer manifestação de vontade, livre, específica e informada, nos termos da qual o titular aceita que os seus dados sejam objeto de tratamento, o qual deve ser obtido através de uma "declaração de consentimento informado " onde seja utilizada uma linguagem clara e acessível.

Nos termos do artigo 10.º da LPD, a declaração de consentimento tem de conter a identificação do responsável pelo tratamento e a finalidade do tratamento, devendo ainda conter informação sobre a existência e as condições do direito de acesso e de retificação por parte do respetivo titular.

Os titulares dos dados, de acordo com a declaração de consentimento informado junta aos autos, apõem as suas assinaturas na mesma, deste modo satisfazendo as exigências legais.

Cabe ao Investigador assegurar a confidencialidade dos dados pessoais e da informação tratada, conforme o estatuído na alínea *g*) do artigo 10.º da Lei n.º 21/2014, de 16 de abril (Lei da Investigação Clínica).

A informação tratada é recolhida de forma lícita (art.º 5.º, n.º1 alínea a) da Lei n.º 67/98), para finalidades determinadas, explícitas e legítimas (cf. alínea b) do mesmo artigo) e não é excessiva.

No que respeita à recolha da raça, o responsável pelo tratamento justifica a sua necessidade invocando o seguinte:

" *(...)a consideração e a recolha do dado "Grupo Racial" dos participantes incluídos no estudo supracitado (campo 4 da ficha do participante) é de essencial relevância científica em prol dos objetivos do mesmo.*
De facto, é hoje consensual que existe variabilidade inter-racial no background genético dos indivíduos condicionando, portanto, a distribuição e a frequência dos polimorfismos genéticos. Sendo este um estudo que visa avaliar o impacto dos polimorfismos genéticos na farmacoterapia/resposta antidepressiva, isto significa que poderemos observar variações na resposta antidepressiva que sejam características

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de um determinado grupo racial mas não de outro. Consequentemente, este facto pode definir diferenças nas intervenções terapêuticas a adotar mediante o grupo racial. Estas considerações são suportadas por inúmeras evidências científicas publicadas na literatura, de entre as quais, saliento a de Eleanor Murphy et al. (2013) (http://www.discoverymedicine.com/Eleanor-Murphy/2013/08/26/pharmacogenetics-of-antidepressants-mood-stabilizers-and-antipsychotics-in-diverse-human-populations/). Assim, a identificação e a caracterização destas correlações está dependente da recolha desta informação por cada participante. Além do mais, o grupo racial pode revelar-se um importante fator de confusão na tentativa de compreender a importância da farmacogenética na individualização de esquemas terapêuticos considerando uma abordagem populacional, exigindo um processo de análise dos dados estratificados por esta variável.

Portanto, apesar de existirem constrangimentos legais a este nível, a recolha desta característica individual dos participantes é totalmente justificada no âmbito do estudo clínico proposto, uma vez que permitirá levar a cabo uma avaliação farmacométrica mais robusta e realista para os três antidepressivos considerados."

Deste modo, pelas razões *supra* invocadas, entende a CNPD que é legítima a recolha e tratamento do dado raça.

O fundamento de legitimidade é o consentimento expresso do titular dos dados.

III. Conclusão

Assim, nos termos das disposições conjugadas do n.º 2 do artigo 7.º, n.º1 do artigo 27.º, al. a) do n.º 1 do artigo 28.º e artigo 30.º da Lei de Proteção de Dados, com as condições e limites fixados na referida Deliberação n.º 227/2007, que se dão aqui por reproduzidos e que fundamentam esta decisão, autoriza-se o tratamento de dados supra referido, para a elaboração do presente estudo, consignando-se o seguinte:

Responsável pelo tratamento: Paulo Ricardo Machado Magalhães;

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Finalidade: Estudo "Avaliação Farmacométrica do Impacto Clínico de Polimorfismos Genéticos do Citocromo P450 e da Glicoproteína-P na Farmacodinamia de Antidepressivos: Fluoxetina, Paroxetina e Venlafaxina";

Categoria de Dados pessoais tratados: código do participante; dados sócio-demográficos (sexo, idade, raça), historial clínico e farmacoterapêutico; severidade do transtorno depressivo; grau de eficácia do tratamento; efeitos adversos da medicação; amostra de sangue; concentrações plasmáticas de fármacos; informação genética.

Formas de exercício do direito de acesso e retificação: Junto do médico assistente.

Interconexões de tratamentos: Não há.

Transferências de dados para países terceiros: Não há.

Prazo de conservação: A chave de codificação dos dados do titular deve ser destruída um mês após o fim do estudo.

Dos termos e condições fixados na Deliberação n.º 227/ 2007 e na presente Autorização decorrem obrigações que o responsável deve cumprir. Deve, igualmente, dar conhecimento dessas condições a todos os intervenientes no circuito de informação.

Lisboa, 14 de outubro de 2014

Filipa Calvão (Presidente)

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APPENDIX C – DETAILED FINDINGS OF THE *GnG-PK/PD-AD* STUDY

Table C.1 Summary of the relevant statistical findings of the primary bivariate analysis involving the independent variables of the *GnG-PK/PD-AD* study.

Bivariate interaction	**Statistical test**	**Descriptive (mean ± standard deviation, median) and post-hoc statistics**
Antidepressant drug ↔ Gender	χ^2 (2) = 10.835, p = 0.004, Cramer´s V = 0.244	Male ↔ VEN (20 out of 33 subjects, AR = 2.7) Female ↔ FLU (73 out of 149 subjects, AR = 3.2)
Duration of treatment ↔ Gender	χ^2 (3) = 9.017, p = 0.029, Eta = 0.223	Male ↔ 2-6 months (11 out of 33 subjects, AR = 2.6) Female ↔ > 24 months (86 out of 149 subjects, AR = 2.5)
Duration of treatment ≠ Gender	U = 3.054, z = 2.732, p = 0.006	↑ Female (median, > 24 months) → Male (median, 12-24 months)
Antidepressant drug ↔ Reason of discontinuation	χ^2 (4) = 10.767, p = 0.029; Cramer´s V = 0.236	VEN ↔ Therapeutic failure (36 out of 66 patients, AR = 2.3)
Duration of depression ↔ Duration of treatment	rs (179) = 0.646, p < 0.001	↑ (↓) Duration of depression ↔ ↑ (↓) Duration of treatment
Chronic depression ↔ Duration of treatment	χ^2 (3) = 72.172, p < 0.001, Eta = 0.631	Chronic depression ↔ treatment > 24 months (95 out of 134 patients, AR = 8.0) No chronic depression ↔ treatment < 12 months (33 out of 48 subjects, ARs = 5.6 and 4.1 for the 2-6 months and 6-12 months categories)
Number of co-morbidities ↔ Age	r_s (180) = 0.274, p < 0.001	↑ (↓) Number of co-morbidities ↔ ↑ (↓) Age
Number of co-morbidities ≠ Age groups	Kruskal-Wallis χ^2 (2) = 14.183, p = 0.001	↑ Elderly subjects (2.5 ± 1.8, 3) → young adult (no co-morbidities) and adult subjects (1.9 ± 1.6, 2.0) (Dunn's p = 0.005 and 0.028, respectively)

Table C.1 Summary of the relevant statistical findings of the primary bivariate analysis involving the independent variables of the *GnG-PK/PD-AD* study.

Bivariate interaction	**Statistical test**	**Descriptive (mean ± standard deviation, median) and post-hoc statistics**
Number of drugs ↔ Number of co-morbidities	r_s (180) = 0.536, p < 0.001	↑ (↓) Number of drugs ↔ ↑ (↓) Number of co-morbidities
Number of drugs ↔ Age	r_s (180) = 0.262, p < 0.001	↑ (↓) Number of drugs ↔ ↑ (↓) Age
Number of antipsychotic drugs ≠ Previous antidepressant treatment	U = 3488.5, z = - 2.778, p = 0.005	↑ Subjects previously treated with antidepressant drugs (0.3 ± 0.5, 0.0) → subjects who were using antidepressant drugs for the first time (0.1 ± 0.3, 0.0)
P-gp DPI ↔ number of co-morbidities	r_s (180) = - 0.385, p < 0.001	↑ (↓) P-gp DPI ↔ ↓ (↑) number of co-morbidities
CYP2C9-2C19-2D6 DPI ↔ number of co-morbidities	r_s (180) = - 0.275, p < 0.001	↑ (↓) CYP2C9-2C19-2D6 DPI ↔ ↓ (↑) number of co-morbidities
P-gp DPI ↔ number of drugs	r_s (180) = - 0.449, p < 0.001	↑ (↓) P-gp DPI ↔ ↓ (↑) number of drugs
CYP2C9-2C19-2D6 DPI ↔ number of drugs	r_s (180) = - 0.681, p < 0.001	↑ (↓) CYP2C9-2C19-2D6 DPI ↔ ↑ (↓) number of drugs
CYP2D6 DPI ≠ Previous antidepressant treatment	U = 4925.5, z = 2.341, p = 0.019	↓ Previous antidepressant treatment (- 2.3 ± 1.3, - 2.3) → antidepressant drugs for the first time *(*- 1.9 ± 1.0, - 1.5) *
Number of potential antidepressant- drug interactions ↔ number of drugs	r_s (180) = 0.797, p < 0.001	↑ (↓) Number of potential antidepressant drug interactions ↔ ↑ (↓) number of drugs

Table C.1 Summary of the relevant statistical findings of the primary bivariate analysis involving the independent variables of the *GnG-PK/PD-AD* study.

Bivariate interaction	**Statistical test**	**Descriptive (mean ± standard deviation, median) and post-hoc statistics**
Number of potential antidepressant-drug interactions ↔ number of co-morbidities	$r_s(180) = 0.307$, $p < 0.001$	↑ (↓) Number of potential antidepressant-drug interactions ↔ ↑ (↓) number of co-morbidities
CYP2C9 AS ≠ Chronic depression	U = 3783.0, z = 2.076, p = 0.038	↑ Chronic depression (1.7 ± 0.4, 2.0) → no chronic depression (1.6 ± 0.5, 1.8)

[], concentrations; ↑, higher; ↓, lower; ↔, related with; →, compared with; ≠, significantly different from; *ABCB1*, P-glycoprotein gene; AR, adjusted standardized residuals; AS, Activity Score; CAS, combined CYP2C9-CYP2C19-CYP2D6 activity score, CYP, cytochrome P450; DPI, Drugs-Protein Interaction Score; gPH, genotype-predicted phenotype; gEMs, genotype-predicted extensive metabolizers; gIMs, genotype-predicted intermediate metabolizers; gPMs, genotype-predicted poor metabolizers; gUMs, genotype-predicted ultra-rapid metabolizers; P-gp, P-glycoprotein; rs, Spearman´s correlation; U, Mann Whitney test; χ^2, Chi-squared test

Bivariate statistical analyses were carried out according to the purpose, distribution and scales of the variables. Associations and correlations between variables were tested using the Chi-squared test (χ^2) and the Spearman´s correlation (rs), respectively. In positive Chi-squared associations, Cramer's V and Eta values were considered as measures of the strength of association for nominal by nominal and nominal by interval variables, respectively. Only associations/correlations with a strength > 0.2 were reported. Differences were investigated by means of Mann Whitney U (U) and Kruskal-Wallis χ^2 with Dunn´s post-hoc analysis

* Note for reading DPI score: a negative value means that there is potential of the protein to be inhibited, while a positive one means that there is potential of the protein to be induced

Table C.2 Detailed description of medical co-morbidities in the sample (N = 182).

Medical co-morbidities	n (%)
Blood	
Anaemia	2 (1.1)
Cardiovascular	97 (53.3)
Aneurism	1 (0.5)
Angina	2 (1.1)
Arrhythmia	5 (2.7)
Cardiac insufficiency	4 (2.2)
Heart attack history	2 (1.1)
Hypertension	62 (34.1)
Ischemic transitory accident	1 (0.5)
Left ventricular hypertrophy	1 (0.5)
Phlebitis and thrombophlebitis	2 (1.1)
Stroke history	5 (2.7)
Varicose veins	12 (6.6)
Endocrinal, nutritional and metabolic	123 (67.6)
Diabetes	19 (10.4)
Dyslipidaemia	61 (33.5)
Goitre	7 (3.8)
Hyperthyroidism	1 (0.5)
Hypothyroidism	14 (7.7)
Other disorders of the thyroid	8 (4.4)
Overweight and obesity	12 (6.6)
Porphyria	1 (0.5)
Eye and ear	9 (4.9)
Glaucoma	2 (1.1)
Otitis	1 (0.5)
Keratoconus	1 (0.5)
Vertiginous syndrome	5 (2.7)
Gastrointestinal	24 (13.2)
Cholelithiasis	1 (0.5)
Colitis	1 (0.5)
Dyspepsia	1 (0.5)
Esophagitis	3 (1.6)
Gastritis	8 (4.4)
Hernia	3 (1.6)
Irritable bowel syndrome	3 (1.6)
Ulcer	4 (2.2)
Genitourinary	10 (5.5)
Prostate benign hyperplasia	4 (2.2)
Salpingitis	1 (0.5)
Urolithiasis	5 (2.7)
Infectious	3 (1.6)
Tonsillitis	1 (0.5)
Typhoid fever	1 (0.5)
Other	1 (0.5)
Mental and behaviour	38 (20.9)
Alcoholism	1 (0.5)
Anxiety	12 (6.6)
Panic attack	1 (0.5)
Tobacco abuse	5 (2.7)
Others	19 (10.4)
Musculoskeletal and connective tissue	39 (21.4)
Ankylosing spondylitis	3 (1.6)
Fibromyalgia	5 (2.7)
Osteoarthrosis	13 (7.1)
Osteoporosis	7 (3.8)
Rheumatoid arthritis	2 (1.1)
Others	9 (4.9)
Nervous system	15 (8.2)
Epilepsy	1 (0.5)
Insomnia	5 (2.7)
Migraine	6 (3.3)
Multiple sclerosis	1 (0.5)
Sleep apnea	1 (0.5)
Trigeminal neuralgia	1 (0.5)
Respiratory	13 (7.1)
Asthma	9 (4.9)
Rhinitis	1 (0.5)
Sinusitis	3 (1.6)
Skin	
Psoriasis	4 (2.2)
Others	19 (10.4)

n (%), absolute frequency (relative frequency)

Table C.3 Allele frequencies of the *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* genes observed in the sample and summary of the Hardy-Weinberg Equilibrium (HWE) and case-control comparison analysis.

	Genotype frequencies [n (%)[1]]			**HWE analysis**		**Allele frequencies [% (95% CI)][2]**			**Case-control comparison**	
Allelic variant	**No allele**	**Heterozygotes**	**Homozygotes**	**Test**	**p-value**	**Present study**	**Control group [3]**	**MAF (%)**	**Test**	**p-value**
*CYP2C9*1* [wt]	9 (0.05)	62 (34.1)	111 (61.0)	$\chi^2(1) = 0.0105$	0.918	78.0 (73.8-82.3)	80.3 (77.9-82.8)	-	Fisher´s Exact Test= 4.156	0.228
*CYP2C9*2*	142 (78.0)	35 (19.2)	5 (2.7)	Exact	0.159	12.4 (9.0-15.8)	12.4 (10.3-14.4)	12.4		
*CYP2C9*3*	149 (81.9)	32 (17.6)	1 (0.5)	Exact	1	9.3 (6.1-12.0)	7.3 (5.56-8.8)	9.3		
*CYP2C9*6*	181 (99.5)	1 (0.5)	0	Exact	1	0.3 (0.03-0.08)	0.0	0.3		
*CYP2C19*1* [wt]	21 (11.5)	69 (37.9)	92 (50.5)	$\chi^2(1) = 1.656$	0.198	69.5 (64.5-74.0)	63.0 (60.0-66.0)	-	Fisher´s Exact Test= 5.179	0.137
*CYP2C19*2*	142 (78.0)	36 (19.8)	4 (2.2)	Exact	0.303	12.1 (8.5-15.2)	14.5 (12.3-16.7)	12.1		
*CYP2C19*3*	182 (100)	0	0	Exact	1	0	0.0	0.0		
*CYP2C19*5*	182 (100)	0	0	Exact	1	0	0.0	0.0		

Table C.3 Allele frequencies of the *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* genes observed in the sample and summary of the Hardy-Weinberg Equilibrium (HWE) and case-control comparison analysis.

	Genotype frequencies [n (%)[1]]			**HWE analysis**		**Allele frequencies [% (95% CI)][2]**			**Case-control comparison**	
Allelic variant	**No allele**	**Heterozygotes**	**Homozygotes**	**Test**	**p-value**	**Present study**	**Control group [3]**	**MAF (%)**	**Test**	**p-value**
*CYP2C19*17*	122 (67.0)	53 (29.1)	7 (3.8)	$\chi^2(1) = 0.044$	0.834	18.4 (14.4-22.4)	22.4 (19.8-25.0)	18.4		
*CYP2D6*1* [wt]	51 (28.0)	94 (51.6)	37 (20.3)	$\chi^2(1) = 0.174$	0.676	46.2 (41.0-51.3)	40.1 (37.7-42.5)	-	$\chi^2(11) = 17.218$	0.102
*CYP2D6*2*	110 (60.4)	58 (31.9)	14 (7.7)	χ^2 (1) = 1.989	0.117	17.0 (13.2-20.9)	17.0 (15.2-18.9)	23.4 (1584C>G)		
*CYP2D6*3*	180 (98.9)	2 (1.1)	0	Exact	1	0.5 (0.2-1.3)	1.4 (0.9-2.1)	0.5		
*CYP2D6*4*	133 (73.1)	44 (24.2)	5 (2.7)	Exact	0.556	14.6 (11.2-18.5)	19.0 (17.2-21.0)	14.6 (1846G>A)		
*CYP2D6*5*	177 (97.3)	5 (2.7)	0	Exact	1	2.5 (0.9-4.1)	1.6 (1.1-2.4)	-		
*CYP2D6*6*	176 (96.7)	6 (3.3)	0	Exact	1	1.6 (0.3-3.0)	0.9 (0.5-1.5)	1.6		
	131(72.0)	45 (24.7)	6 (3.3)	Exact	0.398	0.8 (0.1-1.8)	2.2 (1.6-3.0)	16.0		

Table C.3 Allele frequencies of the *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* genes observed in the sample and summary of the Hardy-Weinberg Equilibrium (HWE) and case-control comparison analysis.

	Genotype frequencies [n (%)[1]]			**HWE analysis**			**Allele frequencies [% (95% CI)][2]**			**Case-control comparison**	
Allelic variant	**No allele**	**Heterozygotes**	**Homozygotes**	**Test**	**p-value**		**Present study**	**Control group [3]**	**MAF (%)**	**Test**	**p-value**
*CYP2D6*17*	180 (98.9)	2 (1.0)	0	Exact	1		0.5 (0.2-1.3)	0.9 (0.6-1.5)	0.5 (1023C>T)		
*CYP2D6*29*	182 (100)	0	0	Exact	1		0.0	0	0.0		
*CYP2D6*35*	169 (92.9)	12 (6.6)	1 (0.5)	Exact	0.229		3.8 (1.7-5.5)	5.3 (4.3-6.5)	3.8 (31G>A)		
*CYP2D6*41*	149 (81.9)	31 (17.0)	2 (1.1)	Exact	0.671		9.6 (6.6-12.7)	7.5 (6.3-9.0)	9.6		
*CYP2D6*2XN*							2.2 (0.7-3.7)	2.9 (2.2-3.9)			
*CYP2D6*1XN*							0.6 (0.2-1.3)	0.6 (0.3-1.1)			
ABCB1 1236C>T	60 (33.0)	87 (47.8)	35 (19.2)	$\chi^2(1) = 0.055$	0.815	*C*	56.9 (51.8-62.0)	58.4 (55.4-61.5)	43.1	$\chi^2(8) = 4.621$	0.797
						T	43.1 (38.0-48.2)	41.6 (38.5-44.6)			
ABCB1 2677G>T/A	65 (35.7)	82 (45.1)	32(17.6)	$\chi^2(1) = 0.329$	0.052	*G*	58.8 (53.7-63.9)	57.3 (54.2-60.3)	40.4		

Table C.3 Allele frequencies of the *CYP2C9, CYP2C19, CYP2D6* and *ABCB1* genes observed in the sample and summary of the Hardy-Weinberg Equilibrium (HWE) and case-control comparison analysis.

	Genotype frequencies [n (%)[1]]			**HWE analysis**			**Allele frequencies [% (95% CI)][2]**			**Case-control comparison**	
Allelic variant	**No allele**	**Heterozygotes**	**Homozygotes**	**Test**	**p-value**		**Present study**	**Control group [3]**	**MAF (%)**	**Test**	**p-value**
						T	40.4 (35.3-45.5)	41.0 (37.9-44.0)			
	65 (35.7)	**2 (1.1)**	**1 (0.5)**	**Exact**	**0.042**	***A***	**0.8 (0.1-1.8)**	**1.8 (1.0-2.6)**			
ABCB1 3435C>T	59 (32.4)	83 (45.6)	40 (22.0)	$\chi^2(1)$ = 0.880	0.348	*C*	55.2 (50.1-60.4)	51.8 (48.7-54.9)	44.8		
						T	44.8 (39.7-49.9)	48.2 (45.1-51.3)			
ABCB1 rs2032588	**166 (91.2)**	**13 (7.1)**	**3 (1.6)**	**Exact**	**0.007**	*G*	94.8 (92.5-97.1)	93.0 (91.5-94.6)	5.2		
						A	5.2 (2.9-7.5)	7.0 (5.4-8.5)			

ABCB1, P-glycoprotein gene; CYP, cytochrome P450; MAF, minor allele frequency; SNP, single nucleotide polymorphism; n (%), Absolute frequency (relative frequency); χ2, chi-squared test; wt, wild-type allele
[1] N = 182; [2] n = total frequency of the allelic variants observed for this gene; [3] 1000 Genomes Project (N = 503) for the CYP2C9, CYP2C19 and ABCB1 and Naranjo *et al.* 2016 (N = 805) for the CYP2D6 (172,184).

Table C.4 Prevalence of depressive symptoms/features evaluated by the 17-item Hamilton Depression Rating Scale (HAMD) (N = 182).

	n (%)
1- Depressed mood	110 (60.4)
2- Guilt	63 (34.6)
3- Suicide	56 (30.8)
4- Initial insomnia	89 (48.9)
5- Middle insomnia	92 (50.5)
6- Delayed insomnia	99 (54.4)
7- Work and interests	97 (53.3)
8- Retardation	41 (22.5)
9- Agitation	49 (26.9)
10- Psychic anxiety	120 (65.9)
11- Somatic anxiety	125 (68.7)
12- Gastrointestinal somatic symptoms	46 (25.3)
13- General somatic symptoms	119 (65.4)
14- Genital symptoms	101 (55.5)
15- Hypochondriasis	77 (42.3)
16- Loss of insight	26 (14.3)
17- Loss of weight	45 (24.7)

n (%), absolute frequency (relative frequency)

Table C.5 Frequencies of endorsement on the *Antidepressant Side-Effect Checklist* (ASEC). Absolute frequency (relative frequency, %), N = 182.

Symptom	0- Absent	1- Mild	2- Moderate	3- Severe	Linked	Relevant
Dry mouth	56 (30.8)	34 (18.7)	54 (29.7)	38 (20.9)	69 (37.9)	58 (31.9)
Drowsiness	91 (50.0)	32 (17.6)	43 (23.6)	16 (8.8)	44 (24.2)	31 (17.0)
Insomnia	100 (54.9)	21 (11.5)	33 (18.1)	28 (15.4)	9 (4.9)	7 (3.8)
Blurred vision	109 (59.9)	33 (18.1)	26 (14.3)	14 (7.7)	21 (11.5)	12 (6.6)
Headache	88 (48.4)	34 (18.7)	42 (23.1)	18 (9.9)	21 (11.5)	13 (7.1)
Constipation	111 (61.0)	14 (7.7)	33 (18.1)	24 (13.2)	26 (14.3)	23 (12.6)
Diarrhoea	157 (86.3)	13 (7.1)	7 (3.8)	5 (2.7)	7 (3.8)	5 (2.7)
Increased appetite	119 (65.4)	20 (11.0)	25 (13.7)	18 (9.9)	26 (14.3)	19 (10.4)
Decreased appetite	130 (71.4)	18 (9.9)	29 (15.9)	5 (2.7)	24 (13.2)	16 (8.8)
Nausea or vomiting	151 (83.0)	17 (9.3)	6 (3.3)	8 (4.4)	12 (6.6)	4 (2.2)
Problems with urination	160 (87.9)	6 (3.3)	9 (4.9)	7 (3.8)	6 (3.3)	5 (2.7)
Problems with sexual function	89 (48.9)	11 (6.0)	34 (18.7)	48 (26.4)	57 (31.3)	51 (28.0)
Palpitations	88 (48.4)	33 (18.1)	44 (24.2)	17 (9.3)	23 (12.6)	18 (9.9)

Table C.5 Frequencies of endorsement on the *Antidepressant Side-Effect Checklist* (ASEC). Absolute frequency (relative frequency, %), N = 182.

Symptom	**0- Absent**	**1- Mild**	**2- Moderate**	**3- Severe**	**Linked**	**Relevant**
Feeling light-headed upon standing	89 (48.9)	40 (22.0)	44 (24.2)	9 (4.9)	30 (16.5)	17 (9.3)
Feeling like the room is spinning	106 (58.2)	31 (17.0)	38 (20.9)	7 (3.8)	18 (9.9)	13 (7.1)
Sweating	106 (58.2)	27 (14.8)	31 (17.0)	18 (9.9)	16 (8.8)	12 (6.6)
Increased body temperature	131 (72.0)	19 (10.4)	19 (10.4)	13 (7.1)	11 (6.0)	7 (3.8)
Tremor	103 (56.6)	27 (14.8)	37 (20.3)	15 (8.2)	28 (15.4)	19 (10.4)
Disorientation	139 (76.4)	23 (12.6)	12 (6.6)	8 (4.4)	18 (9.9)	11 (6.0)
Yawning	75 (41.2)	38 (20.9)	42 (23.1)	27 (14.8)	36 (19.8)	24 (13.2)
Weight gain	97 (53.3)	24 (13.2)	34 (18.7)	27 (14.8)	39 (21.4)	31 (17.0)

Table C.6 Summary of the relevant statistical findings of the bivariate analysis for the subpopulation of patients treated with FLU.

Impact on concentrations		
Bivariate interactions	**Statistical test**	**Descriptive (mean ± standard deviation, median) and post-hoc statistics**
NFLU/FLU ↔ CYP2D6 AS	r_s (74) = 0.568, p < 0.001	↑ (↓) NFLU/FLU ↔ ↑ (↓) CYP2D6 AS
[FLU] ≠ *ABCB1 1236 C>T* genotypes	Kruskal-Wallis χ^2 (2) = 7.398, p = 0.025	↓ in *TT* genotype → *CT* and *CC* genotypes (Dunn's p = 0.08 and 0.026)
[NFLU] ≠ CYP2D6 gPH	F (3, 74) = 4.886, p = 0.004	↑ in CYP2D6 gUMs → gPMs and gIMs, respectively (Tukey post-hoc p = 0.004 and 0.033)
NFLU/FLU ≠ CYP2D6 gPH	Welch's F (3, 9.264) = 9.611, p = 0.003	↑ in CYP2D6 gEMs → gPMs and gIMs, respectively (Games-Howell post-hoc p = 0.001 and 0.18)
NFLU/FLU ≠ FLU-NFLU gPH	Kruskal-Wallis χ^2 (2) = 9.337, p = 0.009	↑ in increased metabolic capacity of FLU-NFLU → decreased metabolic capacity (Dunn's p = 0.021)
[NFLU] ≠ presence/no presence of cardiovascular co-morbidities	t (76) = 2.438, p = 0.017	↓ in patients with cardiovascular co-morbidities (4.4 ± 2.3, 4.1) → no cardiovascular co-morbidities (6.0 ± 3.2, 5.9)
[NFLU] ≠ presence/no presence of endocrinal, nutritional and metabolic co-morbidities	t (76) = 2.706, p = 0.008	↓ in patients with endocrinal, nutritional and metabolic co-morbidities (4.3 ± 2.1, 4.2) → no endocrinal, nutritional and metabolic co-morbidities (6.0 ± 3.2, Md = 5.9)
[FLU] ≠ number of potential antidepressant-drug interactions	Kruskal-Wallis χ^2 (2) = 7.132, p = 0.028	↑ in ≥ 5 antidepressant-drug interactions (5.4 ± 3.0, 5.5) → no antidepressant-drug interactions (3.4 ± 2.6, 3.3) (Dunn's p = 0.010).
NFLU/FLU ≠ number of potential antidepressant-drug interactions	Kruskal-Wallis χ^2 (2) = 12.826, p = 0.002	↓ in ≥ 5 antidepressant-drug interactions (1.1 ± 0.6, 1.0) → 1-4 (1.8 ± 1.6, 1.3) and no AD-drug interactions (3.1 ± 3.3, 2.1) (Dunn's p < 0.001 for both).

Table C.6 Summary of the relevant statistical findings of the bivariate analysis for the subpopulation of patients treated with FLU.

Impact on the therapeutic outcomes		
Bivariate interactions	**Statistical test**	**Descriptive (mean ± standard deviation, median) and post-hoc statistics**
HAMD ↔ CYP2D6 DPI	r_s (77) = - 0.230, p = 0.042	↑ (↓) HAMD ↔ ↓ (↑) CYP2D6 DPI *
Therapeutic remission ↔ CYP2C9 DPI	r_s (77) = - 0.242, p =0.032	↑ (↓) Therapeutic remission ↔ ↓ (↑) CYP2C9 DPI *
ASEC-GARSI ≠ *ABCB1 1236 C>T* genotypes	F (2, 76) = 3.841, p = 0.026	↑ in *CC* (1.0 ± 0.6, 1.1) and *TT* genotypes (1.0 ± 0.5, 1.0) → *CT* genotype (0.70 ± 0.4, 0.6)
ASEC-GARSI ≠ ABCB1 *2677G>T/A* genotypes	F (4, 74) = 2.876, p = 0.028	↑ in *GG* (0.9±0.6, 1.0) and *TT* (1.1±0.5, 1.1) genotypes → *GT* (0.7 ± 0.4, 0.6) genotype
ASEC-PSEDI ≠ ABCB1 *2677G>T/A* genotypes	F (4, 71) = 3.501, p = 0.011	↑ in *GG* (2.0 ± 0.4, 2.0) and *TT* (2.1 ± 0.4, 2.1) genotypes → *GT* (1.7 ± 0.5, 1.7) genotype
ASEC-GARSI ≠ *TTT* haplotype	F (2, 76) = 4.381, p = 0.016	↑ in *TTT-TTT* (1.1 ± 0.5, 1.0) and non-*TTT* (1.0 ± 0.5, 1.0) haplotypes → *TTT* haplotype (0.7 ± 0.4, 0.6) (Tukey p = 0.031)
ASEC-PSEDI ≠ *TTT* haplotype	F (2, 76) = 4.381, p = 0.016	↑ in *TTT-TTT* (2.1 ± 0.4, 2.1) and non-*TTT* (2.0 ± 0.3, 2.0) haplotypes → *TTT* haplotype (1.7 ± 0.5, 1.7) (Tukey p = 0.038)
ASEC-PSER ≠ CYP2C9 phenotypes	U = 524.0, z = - 2.343, p = 0.019	↑ in CYP2C9 gIMs (3.1 ± 2.9, 2.0) → CYP2C9 gEMs (1.9 ± 3.0, 1.0)
HAMD ≠ presence/no presence of nervous system co-morbidities	t (77) = - 2.388, p = 0.019	↑ in patients with nervous system co-morbidities (18.1 ± 6.3, 18.0) → no nervous system co-morbidities (12.6 ± 7.0, 12.0)

Table C.6 Summary of the relevant statistical findings of the bivariate analysis for the subpopulation of patients treated with FLU.

Impact on the clinical outcomes		
Bivariate interactions	**Statistical test**	**Descriptive (mean ± standard deviation, median) and post-hoc statistics**
ASEC-GARSI ≠ presence/no presence of nervous system co-morbidities	t (77) = - 2,309, p = 0.024	↑ in patients with nervous system co-morbidities (1.2 ± 0.5, 1.1) → no nervous system co-morbidities (0.9 ± 0.5, = 0.9)
ASEC-PSEDI ≠ presence/no presence of nervous system co-morbidities	t (77) = - 2,345, p = 0.022	↑ in patients with nervous system co-morbidities (2.2 ± 0.3, 2.2) → no nervous system co-morbidities (1.8 ± 0.4, 1.8)

[], concentrations; ↑, higher; ↓, lower; ↔, related with; →, compared with; ≠, significantly different from; *ABCB1*, P-glycoprotein gene; AS, Activity Score; CYP, cytochrome P450; DPI, Drugs-Protein Interaction Score; gPH, genotype-predicted phenotype; gEMs, genotype-predicted extensive metabolizers; gIMs, genotype-predicted intermedium metabolizers; gPMs, genotype-predicted poor metabolizers; gUMs, genotype-predicted ultra-rapid metabolizers; P-gp, P-glycoprotein.

No differences in the statistical outcomes were verified when the gPMs and gIMs were analysed together in the same group compared to the individual analysis.

* Note for reading DPI score: a negative value indicates that there is potential of the protein to be inhibited, while a positive value indicates that there is potential of the protein to be induced.

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