Clinical Implications of Pharmacogenetics in Psychiatric Treatment

Submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy

Luana Mifsud Buhagiar

Department of Pharmacy
2019

L-Università ta' Malta

FACULTY/INSTITUTE/CENTRE/SCHOOL Medicine and Surgery

DECLARATION OF AUTHENTICITY FOR DOCTORAL STUDENTS

Student's I.D. /Code 392288M

Student's Name & Surname Luana Mifsud Buhagiar

Course Doctor of Philosophy

Title of Dissertation/Thesis

Clinical Implications of Pharmacogenetics in Psychiatric Treatment

(a) Authenticity of Thesis/Dissertation

I hereby declare that I am the legitimate author of this Thesis/Dissertation and that it is my original work.
No portion of this work has been submitted in support of an application for another degree or qualification of this or any other university or institution of higher education.
I hold the University of Malta harmless against any third party claims with regard to copyright violation, breach of confidentiality, defamation and any other third party right infringement.

(b) Research Code of Practice and Ethics Review Procedure

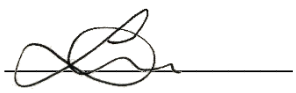
I declare that I have abided by the University's Research Ethics Review Procedures.

☑ As a Ph.D. student, as per Regulation 49 of the Doctor of Philosophy Regulations, I accept that my thesis be made publicly available on the University of Malta Institutional Repository.

☐ As a Doctor of Sacred Theology student, as per Regulation 17 of the Doctor of Sacred Theology Regulations, I accept that my thesis be made publicly available on the University of Malta Institutional Repository.

☐ As a Doctor of Music student, as per Regulation 24 of the Doctor of Music Regulations, I accept that my dissertation be made publicly available on the University of Malta Institutional Repository.

☐ As a Professional Doctorate student, as per Regulation 54 of the Professional Doctorate Regulations, I accept that my dissertation be made publicly available on the University of Malta Institutional Repository.



Signature of Student

LUANA MIFSUD BUHAGIAR

Name of Student (in Caps)

3 October 2019

Date

Ben, my youngest champion, all for you

To

Prof Godfrey LaFerla
Dean, Faculty of Medicine and Surgery, University of Malta; *principal supervisor*

Prof Anthony Serracino Inglott
Professor, Department of Pharmacy, University of Malta; *co-supervisor*

Dr Anton Grech
Clinical Chairman, Department of Psychiatry, Ministry of Health, Malta; *advisor*

Dr Marilyn Casha, Dr Christopher Barbara, Mr Carmel Sammut, Dr Robert Xuereb, Dr Marisa Cassar, Dr Janis Vella Szijj, Dr Nicolette Sammut Bartolo, and Prof Liberato Camilleri

Management and personnel at the Malta Medicines Authority, Mater Dei Hospital, and the Department of Pharmacy, Faculty of Medicine and Surgery, University of Malta

I take pride in acknowledging the treasured inspiration, relentless engagement and commitment endowed by all towards the realisation of this work.
The opportunity to pursue my research, blessed by prolific guidance and empathic support, bestowed an intense experience of learning and personal growth.
Thank you for being part of this PhD journey in such a significant way.
No statistical data is attached for the latter; on just this, you may wish to essentially trust my words.

This research work was funded by the ENDEAVOUR Scholarships Scheme.

Table of Contents

List of Tables vi
List of Figures vii
List of Abbreviations viii
Definition of terms xi
Abstract xiv

Chapter 1
Introduction 1
1.1 Integrative Overview 2
1.1.1 Genetics, genomics and pharmacogenetics 2
1.1.2 The role of genes in psychiatric disorders 4
1.1.3 Pharmacological management of depression 6
1.1.4 Efficacy and tolerability of antidepressants 9
1.1.5 Tricyclic antidepressants for neuropathic pain 12
1.1.6 Therapeutic drug monitoring for amitriptyline 15
1.1.7 Genetic polymorphisms concerning amitriptyline metabolism 20
1.1.8 Clinical relevance of pharmacogenetics in amitriptyline therapy 24
1.1.9 Regulatory sciences perspective 31
1.1.10 The local scenario 40
1.2 Outline and rationale 43
1.3 Research questions, aims and objectives 47

Chapter 2
Methodology 48
2.1 Research design 49
2.2 Regulatory framework 51
2.2.1 Contextual background and data sources 51
2.2.2 Pharmacogenetic considerations in the literature of amitriptyline products 57
2.2.3 Amitriptyline safety signals, causality assessment and case study reports 58
2.3 Analytical approach 62
2.3.1 Purpose and resources 62
2.3.2 Laboratory analysis of the amitriptyline products available in Malta 63
2.3.3 High-performance liquid chromatography experimentation 64
2.3.4 LC-MS/MS method development 70
2.3.5 Genomic DNA extraction and CYP450 genotyping 74
2.4 Clinical investigation 78
2.4.1 Scope and practice setting 79
2.4.2 Ethical considerations 80
2.4.3 Recruitment protocol 82
2.4.4 Sample collection 85
2.4.5 Patient assessment 86
2.5 Data processing 89

Chapter 3
Results 95
3.1 Regulatory inferences 96
3.1.1 Harmonisation of the amitriptyline Summary of Product Characteristics 96
3.1.2 Safety appraisals: amitriptyline interactions and adverse reaction reports 101
3.2 Analytical developments 109
3.2.1 High performance liquid chromatography 109
3.2.2 Liquid chromatography tandem mass spectrometry 119
3.2.3 *CYP2C19* and *CYP2D6* genotypic measures 127
3.3 Clinical observations 131
3.3.1 Patient population demographics 131
3.3.2 Genotype, metaboliser status and concomitant drugs 132
3.3.3 Serum levels of parent drug and metabolites during amitriptyline therapy 135
3.3.4 CYP450 enzymes, serum concentrations and side-effects 154
3.4 Abridgement 161

Chapter 4
Discussion 164
4.1 Synthesis of the literature and principal findings 165
4.2 Methodological critique and study limitations 189
4.3 Practical implications and future directions 195
4.4 Conclusion 206

References 208

List of Publications and Abstracts 229

Appendices 231
A. Permission from the Journal of the Brazilian Chemistry Society for Figure 1-1 231
B. Genomic DNA extraction and CYP450 genotyping experimentation 233
C. University Research Ethics Committee (UREC) Approval 239
D. Patient Information Sheets 241
E. Patient Consent Forms 246
F. Data Collection Forms 251

List of Tables

Table 1-1: *CYP2C19* and *CYP2D6* variants 23
Table 1-2: Clinical Pharmacogenetics Implementation Consortium (CPIC) dosing recommendation for amitriptyline based on CYP2D6 and CYP2C19 phenotypes 27
Table 1-3: Guidance documents issued by the European Medicines Agency in relation to pharmacogenomics 36
Table 2-1: Single Nucleotide Polymorphisms detected by Mutector™ kits 76
Table 3-1: Assessment of 'Drug Interaction' signal for amitriptyline 104
Table 3-2: Individual Case Study Reports for amitriptyline and *dry mouth* or *sedation* 108
Table 3-3: LC-MS/MS – Retention times, Multiple Reaction Monitoring (MRM) transitions and optimized parameters 119
Table 3-4: LC-MS/MS – Linearity and recovery 121
Table 3-5: LC-MS/MS – Intra- and inter-day precision and bias 121
Table 3-6: LC-MS/MS – Matrix effects at the limits of quantification 122
Table 3-7: Amitriptyline and nortriptyline serum concentrations, as determined by the method developed in-house and by the out-sourced lab 124
Table 3-8: Test of normality for measured concentrations 125
Table 3-9: Test of correlation between amitriptyline and nortriptyline concentrations determined by in-house method and concentrations reported by the out-sourced lab 126
Table 3-10: Lab-reported CYP2C19 and CYP2D6 genotyping results for the recruited patients 128
Table 3-11: Genotype-inferred *CYP2D6* activity scores and metaboliser status for recruited patients, updated in line with the 2019 Consensus on CYP2D6 genotype to phenotype 130
Table 3-12: Distribution of alleles in recruited patients, and corresponding activity 133
Table 3-13: CYP2C19 and CYP2D6 metaboliser status characterised in sample population 133
Table 3-14: Patient sample included in evaluation of serum levels 135
Table 3-15: Test of correlation between amitriptyline daily dose and serum concentrations of parent compound, and its desmethylated and hydroxylated metabolites 136
Table 3-16: Generalised Linear Model – nortriptyline to amitriptyline concentration ratio 138
Table 3-17: Concentrations of hydroxy-metabolites – descriptive statistics and correlations 140
Table 3-18: Generalised Linear Model – hydroxy-amitriptyline to amitriptyline ratio 140
Table 3-19: Generalised Linear Model – hydroxy-nortriptyline to nortriptyline ratio 141
Table 3-20: Difference in measured Ct from expected amitriptyline + nortriptyline range, according to risk of CYP2D6 inhibition by concomitant drug(s) 142
Table 3-21: Logistic regression model – Measured Ct being below/within/above the expected amitriptyline + nortriptyline range 143
Table 3-22: Cmin of patients, as compared to expected range 143
Table 3-23: Relation between normalised amitriptyline Cmin concentration and risk of CYP2D6 inhibition by concomitant drug(s) 144
Table 3-24: Relation between normalised nortriptyline Cmin concentration and risk of CYP2D6 inhibition by concomitant drug(s) 145
Table 3-25: Relation between normalised amitriptyline + nortriptyline Cmin concentration and risk of CYP2D6 inhibition by concomitant drug(s) 145
Table 3-26: Generalized linear model – Normalised Cmin amitriptyline, nortriptyline and amitriptyline + nortriptyline 146
Table 3-27: Relation between normalised amitriptyline + nortriptyline Cmin concentration and revised CYP2D6 status 147
Table 3-28: Genotype-guided dosing recommendations, considering serum levels and concomitant CYP2C19/CYP2D6 inhibitors, for recruited patients under psychiatric care 152
Table 3-29: Dry mouth score according to amitriptyline + nortriptyline concentration 158

List of Figures

Figure 1-1: Main metabolic pathways of amitriptyline 18
Figure 2-1: Synopsis of methodology 50
Figure 2-2: Periodic Safety Update Report (PSUR) Single Assessment (PSUSA) procedure for nationally authorised products 55
Figure 2-3: EU signal management process for nationally authorised products 55
Figure 2-4: Data extraction for assessment of drug interaction cases 59
Figure 2-5: Sample results for *CYP2D6* and *CYP2C19* genotyping with Mutector™ kits (Trimgen) 77
Figure 2-6: *CYP2D6* Tube A genotyping results (Trimgen) 77
Figure 2-7: Recruitment of patients on amitriptyline, under psychiatric care 83
Figure 3-1: Reports stratified by year of submission and seriousness 106
Figure 3-2: Outcome of causality assessment per number of cases 107
Figure 3-3: HPLC – Plots of the retention time vs mobile phase acetonitrile percentage 111
Figure 3-4: HPLC – Speciation plot for amitriptyline 111
Figure 3-5: HPLC – Separation of amitriptyline, nortriptyline and clomipramine 112
Figure 3-6: HPLC – Calibration curves for amitriptyline and nortriptyline 114
Figure 3-7: HPLC – Chromatogram showing separation of amitriptyline, nortriptyline and their hydroxy-metabolites, with clomipramine as internal standard 115
Figure 3-8: HPLC – Chromatogram for serum spiked with 100 µg/mL amitriptyline and clomipramine 116
Figure 3-9: HPLC – Chromatogram for serum spiked with 1 µg/mL amitriptyline and 100 µg/mL clomipramine 117
Figure 3-10: HPLC – Comparison between chromatogram for amitriptyline standard solution and chromatogram for serum spiked with amitriptyline standard solution 118
Figure 3-11: UHPLC – Chromatogram using Ultra-High Performance Liquid Chromatography system 118
Figure 3-12: LC-MS/MS – Filtered Multiple Reaction Monitoring (MRM) ion chromatograms 120
Figure 3-13: LC-MS/MS – Calibration curves 122
Figure 3-14: Distribution of amitriptyline and nortriptyline concentrations for which both in-house and out-sourced results were made available 125
Figure 3-15: Amitriptyline concentrations measured in-house vs reported by out-sourced lab 126
Figure 3-16: Nortriptyline concentrations measured in-house vs reported by out-sourced lab 127
Figure 3-17: Distribution of genotypic categories in sample population 134
Figure 3-18: Correlation between the daily dose of amitriptyline and serum levels of amitriptyline and metabolites (AMI, NOR, E-10-OH AMI, Z-10-OH AMI, E-10-OH NOR, and Z-10-OH NOR) 137
Figure 3-19: Nortriptyline to amitriptyline concentration ratio as related to CYP2C19 139
Figure 3-20: Variation in amitriptyline + nortriptyline normalised Cmin among patients 149
Figure 3-21: Correlations between hydroxy-metabolites to parent concentration ratios and *CYP2D6* activity score 150
Figure 3-22: Preliminary assessment of ECG measures – QT correction 156
Figure 3-23: Dry mouth reports according to measured amitriptyline + nortriptyline concentration in relation to the expected dose-related reference range 158

List of Abbreviations

ACCP	American College of Clinical Pharmacy
ADR	Adverse drug reaction
AMI	Amitriptyline
ASEC	Antidepressant Side Effects Checklist
BDNF	Brain derived neurotrophic factor
CDS	Clinical decision support
CDx	Companion diagnostic
CHMP	Committee for Medicinal Products for Human Use
CIOMS	Council of International Organisation of Medical Sciences
CLIA	Clinical Laboratory Improvement Amendments
CMDh	Coordination Group for Mutual Recognition and Decentralised Procedure - human
Cmin	Trough concentration
CNS	Central nervous system
CNV	Copy number variation
CPIC	Clinical Pharmacogenetics Implementation Consortium
Ct	Concentration at time of blood withdrawal
CV	Coefficient of variation
CYP	Cytochrome P450 CYP enzyme is referred to in standard format (e.g. CYP2D6) and its associated gene is designated by italics (e.g. *CYP2D6*)
DPWG	Dutch Pharmacogenetics Working Group
EC	European Commission
EDQM	European Directorate for the Quality of Medicines
EHR	Electronic Health Record
EMA	European Medicines Agency
E-OH AMI	E-10-hydroxyamitriptyline
E-OH NOR	E-10-hydroxynortriptyline
eRMR	Electronic Reaction Monitoring Report
IS	Internal standard
EUnetHTA	European Network for Health Technology Assessment
EURD	EU reference date
EVDAS	EudraVigilance Data Analysis System
FAERS	FDA Adverse Event Reporting System
FDA	Food and Drug Administration (US)
GENDEP	Genome-based therapeutic drugs for depression
HPLC	High performance liquid chromatography

HTA	Health technology assessment
ICH	International Conference on Harmonisation
ICSR	Individual case safety report
IVD	In-vitro diagnostics
LC	Liquid chromatography
LLOQ	Lower limit of quantification
LOD	Limit of detection
MADRS	Montgomery Asberg Depression Rating Scale
MAH	Marketing Authorisation Holder
MAOIs	Monoamine-oxidase inhibitors
MCH	Mount Carmel Hospital
MDH	Mater Dei Hospital
MF	Matrix factor
MHRA	Medicines and Healthcare Products Regulatory Agency
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NAP	Nationally authorised product
NCA	National competent authority
NaSSAs	Noradrenergic and specific serotonin antidepressants
NICE	National Institute for Health and Clinical Excellence
NOR	Nortriptyline
NRIs	Noradrenaline re-uptake inhibitors
PCR	Polymerase chain reaction
PD-Q	painDETECT questionnaire
PI	Prescribing information
PgWP	Pharmacogenomics Working Party
PGx	Pharmacogenomics
PMDA	Pharmaceuticals and Medical Devices Agency
POP	Psychiatric out-patients
POYC	Pharmacy of Your Choice
PRAC	Pharmacovigilance Risk Assessment Committee
PREPARE	Preemptive Pharmacogenomic Testing for Preventing Adverse Drug Reactions
PRR	Proportional reporting ratio
PSUR	Periodic Safety Update Report
PSUSA	PSUR Single Assessment
QC	Quality control

QTc	Corrected QT interval
r^2	Coefficient of determination
RNPGx	French national network of pharmacogenetics
SAWP	Scientific Advice Working Party
SDR	Signal of disproportionate reporting
SmPC	Summary of product characteristics
SNP	Single nucleotide polymorphism
SNRIs	Serotonin and noradrenaline reuptake inhibitors
SSRIs	Selective serotonin re-uptake inhibitors
STAR*D	Sequenced Treatment Alternatives to Relieve Depression
TCA	Tricyclic antidepressant
TDM	Therapeutic drug monitoring
Trk	Tyrosine kinase receptors
UHPLC	Ultra-high performance liquid chromatography
ULOQ	Upper limit of quantification
U-PGx	Ubiquitous Pharmacogenomics
UREC	University Research Ethics Committee
Z-OH AMI	Z-10-hydroxyamitriptyline
Z-OH NOR	Z-10-hydroxynortriptyline

Definition of terms

Term	*Definition*
Allele	One of two or more versions of a gene. If the two alleles for a gene are the same, the individual is homozygous for that gene. If the alleles are different, the individual is heterozygous. Though the term allele was originally used to describe variation among genes, it now also refers to variation among non-coding DNA sequences. *Citation* Biesecker LG. Talking Glossary of Genetic Terms [Online]. National Human Genome Research Institute; NIH, US [accessed 2019 Jul 31]. Available from: https://www.genome.gov/genetics-glossary/Allele.
Causality assessment	Evaluation of the relationship between particular drug treatment and the occurrence of an adverse event. *Citation* Hire RC, Kinage PJ, Gaikwad NN. Causality assessment in pharmacovigilance: a step towards quality care. *Sch J App Med Sci* 2013;1(5):386-92.
Copy number variation	Two or more copies of the same gene sequence may be inherited from a parent or the gene may be deleted altogether, differing from the expected total value of two gene copies. In some cases, the total copy number may be zero or as high as ten or more. *Citation* Jarvis JP, Prakasam Peter A, Shaman JA. Consequences of CYP2D6 copy number variation for pharmacogenomics in psychiatry. *Front Psychiatry* 2019;10:432. doi:10.3389/fpsyt.2019.00432.
Drug-drug interaction	An interaction (pharmacokinetic/pharmacodynamic) caused by drug response to a co-administered drug. Phenocopying is the result of a drug-drug interaction. For instance, initiating a potent CYP inhibitor results in changing an individual from the genotype normal metaboliser to the phenotype poor metaboliser.
Drug-gene interaction	An interaction caused by drug response to CYP450 genetics. Although usually not deemed as an interaction, the CYP genotype of an individual can result in altered drug concentrations when a medicine is introduced to the treatment regimen.
Drug-drug-gene interaction	An interaction that is a cumulative effect of both a drug-drug interaction and drug-gene interaction. It may occur when an individual taking a drug metabolised by two CYP pathways is given a medication that inhibits or induces one of the CYP pathways, while their genetics alters the metabolism of the other pathway(s). The impact depends upon the fraction of the substrate drug that is eliminated via each pathway. *Citation* Verbeurgt P, Mamiya T, Oesterheld J. How common are drug and gene interactions? Prevalence in a sample of 1143 patients with *CYP2C9*, *CYP2C19* and *CYP2D6* genotyping. *Pharmacogenomics* 2014;15(5):655-65.

Term	*Definition*
Genomic biomarker	A measurable DNA and/or RNA characteristic that is an indicator of normal biologic processes, pathogenic processes, and/or response to therapeutic or other interventions. *Citation* ICH Harmonised Tripartite Guideline E15. Definitions for genomic biomarkers, pharmacogenomics, pharmacogenetics, genomic data and sample coding categories. ICH; 2007.
Genotype	The combination of two alleles at one genomic location (locus) or base pair in an individual. *Citation* Raby BA & Blank RD. Genetics: Glossary of terms [Online]. UpToDate® [accessed 2019 Jul 31]. Available from: https://www.uptodate.com/contents/genetics-glossary-of-terms.
Haplotype & Diplotype	*CYP2D6* and *CYP2C19* genetic variants are typically reported as haplotypes, which are defined by a specific combination of single nucleotide polymorphisms and/or other sequence variants including insertions and deletions that are interrogated by genotype analysis. Haplotypes are assigned a star-allele (*). Laboratories typically report a diplotype, denoting the inherited maternal and paternal star-alleles. *Citation* Hicks JK, Sangkuhl K, Swen JJ, Ellingrod VL, Müller DJ, Shimoda K et al. Clinical pharmacogenetics implementation consortium guideline (CPIC) for CYP2D6 and CYP2C19 genotypes and dosing of tricyclic antidepressants: 2016 update. *Clin Pharmacol Ther* 2017;102(Suppl):S1-S52.
Pharmacogenetics	Pharmacogenetics is a subset of pharmacogenomics and is defined as the study of variations in DNA sequence as related to drug response.
Pharmacogenomics	The study of variations of DNA and RNA characteristics as related to drug response. *Citation* ICH Harmonised Tripartite Guideline E15. Definitions for genomic biomarkers, pharmacogenomics, pharmacogenetics, genomic data and sample coding categories. ICH; 2007.
Pharmacovigilance	The science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other medicine-related problem. *Citation* European Medicines Agency. Pharmacovigilance Overview [Online]. EMA [accessed 2019 Jul 31]. Available from: https://www.ema.europa.eu/en/human-regulatory/overview/pharmacovigilance.
Phenoconversion	The process whereby the predicted genotypic metaboliser status is different from the clinically observed phenotypic metaboliser status as a result of non-genetic factors. For instance, a normal metaboliser genotype can present a clinical response similar to a poor or intermediate metaboliser because of drug-drug interactions. *Citation* Abubakar A & Bentley O. Precision medicine and pharmacogenomics in community and primary care settings. *PharmacyToday* 2018;24(2):55-68.

Term	*Definition*
Phenotype	Broadly defined as observable characteristics of an individual, arising from complex interactions between its genotype and its environment. The observed phenotype may not match the phenotype predicted from genotype data. For instance, measuring the metabolic phenotype of a genotypic normal metaboliser may reveal a phenotypically poor metaboliser due to interaction with other CYP substrates or inhibitors. *Citations* Gkoutos GV, Schofield PN, Hoehndorf R. The anatomy of phenotype ontologies: principles, properties and applications. *Brief Bioinform* 2018;19(5):1008-21. LLerena A, Naranjo ME, Rodrigues-Soares F, Penas-LLedó EM, Fariñas H, Tarazona-Santos E. Interethnic variability of CYP2D6 alleles and of predicted and measured metabolic phenotypes across world populations. *Expert Opin Drug Metab Toxicol* 2014;10:1569–83.
Single nucleotide polymorphism	DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered. SNPs are the most abundant variant in the human genome and the most common source of genetic variation. *Citation* The Journal of the American Medical Association. Glossary of Genomics Terms. *JAMA* 2013; 309(14):1533-5. doi:10.1001/jama.2013.2950.
Therapeutic drug monitoring	The quantification and interpretation of drug concentrations in blood to optimize pharmacotherapy. *Citation* Hiemke C, Bergemann N, Clement HW, Conca A, Deckert J, Domschke K, et al. Consensus guidelines for therapeutic drug monitoring in neuropsychopharmacology: Update 2017. *Pharmacopsychiatry* 2018;51:9–62.

The definitions presented are reproduced from the cited sources, including adaptation of the text, as applicable, to serve the purpose of interpreting the terms in the context of the work within this research.

Abstract

Tricyclic antidepressants have played leading roles in the psychiatric pharmacotherapy scene along the years, with amitriptyline credited in the management of depression, and progressively also in neuropathic pain. Exposure to amitriptyline and its metabolites is influenced by genetic polymorphisms of cytochrome P450 subfamily enzymes, particularly CYP2C19 and CYP2D6. Evaluating the impact of genotype-inferred variability on individual pharmacokinetics and corresponding outcomes, as may be moderated by co-medications and confounding host factors, should enable better informed use of this established drug. This research studied the intricate implications of pharmacogenetics in delivering precision medicine, through an investigative methodology that integrates regulatory, analytical (genetic and chemical), and clinical aspects, using amitriptyline as a case example.

The regulatory aspect comprised the study of (i) pharmacogenetic considerations in the official sources of information for amitriptyline products, by direct inspection of the 33 nationally accessible Summary of Product Characteristics (SmPCs) of amitriptyline products in the UK, and (ii) pharmacogenetic considerations through the evaluation of amitriptyline safety concerns, by systematic extraction of 'drug interaction' reports from EudraVigilance and carrying out causality assessment for the 73 cases involving amitriptyline and one of the CYP2D6 inhibitors/substrates listed in the amitriptyline SmPC. The reported cases of suspected interactions were assessed on whether the effect may be linked to altered enzymatic metabolism caused by co-administration, and scored as highly probable, probable, possible, or unlikely/uncertain. Reporters' reference to potential CYP-mediated variations in metabolism and inclusion, within the reports, of outcomes from corresponding investigations, were reviewed.

The analytical aspect included multiple laboratory settings and technical resources for: (i) measuring serum levels of amitriptyline, nortriptyline, and their hydroxy-metabolites, by applied trialling of sample preparation procedures, and assaying via high performance liquid chromatography, ultra-high performance liquid chromatography, and liquid chromatography tandem mass spectrometry, and (ii) genomic DNA extraction for CYP450 genotyping, by experimenting with buccal swabs for non-invasive sample collection and assaying with TrimGen Mutector™ genotyping kits and TaqMan® SNP Genotyping. An LC-MS/MS method was developed and validated for the simultaneous quantification of amitriptyline, nortriptyline, E- and Z-10-hydroxyamitriptyline, and E- and Z-10-hydroxynortriptyline in human serum. Buccal cells rendered effective sources for extracting DNA and TaqMan® genotyping of *CYP2C19* and *CYP2D6*, including copy number variation analysis for the latter. Following the Clinical Pharmacogenetics Implementation Consortium 2019 consensus of CYP2D6 genotype to phenotype, the metabolizer status inferred by laboratory reports was thenceforth reconsidered accordingly.

The clinical aspect involved investigation of: (i) the influence of genotype, metaboliser status, and the potential of phenoconversion on blood levels, evaluated alongside expected dose-related reference ranges, and (ii) the interplay between CYP2C19 and CYP2D6, therapeutic drug monitoring outcomes and side-effect measures. Following ethics approval from the University Research Ethics Committee, 44 patients attending the pain or psychiatric outpatient clinics at Mater Dei Hospital, for pain or depressive illness sufficiently severe to require therapy, with amitriptyline as monotherapy or as add-on, were recruited upon written, informed consent. Samples of blood and buccal cells were collected for measurement of serum levels and genotyping, while the Antidepressant Side Effects Checklist was used for scoring effects on a four-point scale. Patients underwent

an electrocardiographic examination and the risk of CYP inhibition by concomitant drugs was incorporated in the analyses. Comprehensive data analysis, including computation of dose-related reference ranges for subjects within the cohort, facilitated further investigation. IBM SPSS Statistics® software was used for statistical analysis, using the criterion of $P < 0.05$ for inferring statistical significance. The results are presented as regulatory inferences, analytical developments, and clinical observations.

Regulatory inferences draw attention to the 2017 harmonised amitriptyline SmPC, extending sections on CYP2C19 and CYP2D6, which were found to be updated in 61% of the 33 inspected SmPCs, as accessible from the UK national competent authority. In the causality assessment of 73 suspected 'drug interaction' cases extracted from EudraVigilance, 55 scored as 'possible', implying that the clinical event occurred within a reasonable time sequence to the administration of amitriptyline and the CYP2D6 substrate/inhibitor, but which could also be explained by concurrent disease, and the information on drug(s) withdrawal was lacking or unclear. Reference to CYP enzymatic metabolism and potential inhibition was made in 15% of the reports assessed, whereas only two reporters mentioned genetic testing.

Analytical developments in measuring serum concentrations using high (and ultra-high) performance liquid chromatography, experimenting with both liquid-liquid back extraction and protein precipitation as sample preparation procedures, did not render apposite means for the concentrations anticipated in patient serum samples. In the LC-MS/MS method, E-10-hydroxyamitripyline, E-10-hydroxynortriptyline, Z-10-hydroxyamitriprtline, Z-10-hydroxynortriptyline, amitriptyline, and nortriptyline eluted consecutively within a 6-minute run-time. The method was validated in human serum with a lower limit of quantitation of 0.5 ng/mL for all analytes. A linear response function

was established for the range of concentrations 0.5 – 400 ng/mL ($r^2 > 0.999$). Out of the 44 TaqMan® genotyping assays on DNA extracted from buccal cells, results were available for *CYP2C19* in 43 patients (1 failed), and for *CYP2D6* in 42 patients (1 failed, 1 indeterminate). Aberrant metabolism for CYP2C19 or CYP2D6 was identified in 30% and 17% of subjects respectively. Updating the CYP2D6 metaboliser status in line with the 2019 Consensus renders 50% of patients to potentially deviate from the normal CYP2D6 metaboliser status.

Clinical observations in the recruited patients corroborate a positive correlation between the daily dose of amitriptyline and all measured serum concentrations – amitriptyline, nortriptyline, and their hydroxy-metabolites ($P < 0.01$). CYP2C19 metaboliser status represented the significant main effect in explaining inter-patient variation in the nortriptyline to amitriptyline concentration ratio (ranging between 0.1 and 2.0), with the mean nortriptyline to amitriptyline ratio being 0.2 lower in intermediate metabolisers and 0.6 higher in rapid metabolisers, compared to normal CYP2C19 metabolisers. The mean ratio of hydroxy-metabolites to parent was lower in patients at high risk of CYP2D6 inhibition by concomitant drugs, compared to patients for whom the risk was conceivably inferior, and was significantly positively correlated to the CYP2D6 activity score. In patients at high risk of CYP2D6 inhibition by concomitant drugs, the amitriptyline + nortriptyline concentration was on average 52% above the higher end of the dose-related reference range ($P=0.001$), with CYP2D6 inhibition risk explaining 44% of variation in the measured concentrations of amitriptyline + nortriptyline being below, within, or above the expected range ($P=0.003$).

ECG outcomes are explained in terms of the method of QT correction, providing supporting evidence that compared to Fridericia's formula, Bazett's formula

underestimates at heart rates below 60 bpm and overcorrects QTc values at elevated heart rates. Out of the 44 participants, 2 patients had QTcF prolongation, while PR prolongation and QRS widening were identified in 2 and 4 patients respectively. The results of this research indicate that unlike dry mouth, drowsiness may become less problematic in the long-term, even in case of amitriptyline dose escalation. Genotype-inferred CYP2D6 and CYP2C19 metaboliser status did not render significant correlations to dry mouth or drowsiness scores, and neither to the total side-effect burden. The research findings are discussed through critical engagement with the literature and previous studies in the field.

The fragmentary nature of adverse reaction reports, encompassing limited investigations and follow up data, calls for the refinement of current pharmacovigilance structures to evaluate safety risks conferred by aberrant metabolism. The analytical developments, assessed in practice, coupled to the corollary signalled for the risk of CYP inhibition by concomitant drugs, support present knowledge to inform precision medicine. The integrated approach adopted in the construal of amitriptyline genotype-guided dosing recommendations for subjects under psychiatric care, provides collated evidence to the understanding that due consideration of contributors to a patient's metabolic profile at a point in time may complement the potential acclaimed to the clinical implementation of pharmacogenetics. This multi-aspect work, based on the collection and analysis of original data, serves to stimulate expedient appraisal of evolving theory and experimental research, imparting analytical developments and guiding information, to better understand clinical presentations and translate into significant interventions in practice.

Keywords: amitriptyline; analytical developments; clinical practice; dosing recommendations; genotyping; metabolism; phenoconversion; reference ranges; regulatory vigilance; therapeutic drug monitoring.

Chapter 1
Introduction

1.1 Integrative Overview

The introductory chapter is intended to present the growth of knowledge in the field of genetics and genomics, focusing on the applicability of research findings to practice. The literature review introduces pharmacogenetics, with particular concern for the tricyclic antidepressants, directing attention towards amitriptyline as a case example. Prescribing guided by genotype is considered in light of the pertinent analytical, clinical and regulatory developments. The scientific evidence and central issues identified in the literature inform the subsequent formulation of research questions and the integrated methodological approach adopted to study them.

1.1.1 Genetics, genomics and pharmacogenetics

Human genetic research has progressed from monogenic, with one modifier thought to explain most of the variation observed, to oligogenic, stirring away from single causative genes, to complex inheritance and diseases (Kousi & Katsanis, 2015). By combining "gene" and "ome", a suffix inferring completeness or perhaps appropriated from "chromosome", the term genome was formulated in 1920 by Hans Winkler, a German botanist. The scientific lexicon was enriched with "genomics" in 1987 through the title of a new journal cofounded by Frank Ruddle and Victor McKusick of Johns Hopkins University which compared genomes from different species, focusing on gene mapping and DNA sequencing (Ozdemir et al, 2009).

Beyond the play on words, "omics science" substantively transformed the design, throughput and process of research, from hypothesis-driven to data generation at multiple levels. Omics technologies enabled widespread exploration of the dynamic variability shaping biological networks implicated in the mechanisms of pathophysiological disease

as well as drug safety and efficacy (Wilke et al, 2008). The clinical implications of genetic variance on medicine action hinted at years ago, are realised today as contributors to personalised medicine. The link between response to phenylthiourea and an inherited autosomal recessive trait demonstrated by Larry Snyder is often acclaimed for steering this era (Snyder, 1932). Friedrich Vogel in 1959 gave it a name – pharmacogenetics – defining the 'study of the role of genetics in drug response' (Vogel, 1959).

Preliminary experiments focussed on 'simple' drug-gene interactions and the impact of heritability in plasma drug half-lives. Between 1988 and 1995, the scientific literature reported molecular cloning and identification of defective alleles for the *CYP2D6*, *NAT2*, *CYP2C19* and *TPMT* genes (Gonzalez et al, 1988, Blum et al, 1990, Goldstein & de Morais, 1994, Krynetski et al, 1995). The late 1990s welcomed the single-nucleotide polymorphisms (SNPs) wave, seeking associations between SNPs and multiplex phenotypes, as for the response to clozapine in schizophrenia. Unequivocal genotypes, or even phenotypes, appeared virtually impossible to characterize in real-world populations at that time. Physicians nourished an interest in recognition of disease, discerning "affected" and "unaffected" subjects. Pharmacologists fostered the need to evaluate the quantitative response to a drug which is impacted by multiple genetic and environmental factors (Nebert et al, 2008).

Lack of unequivocal data was unremittingly identified for areas ranging from allelic heterogeneity, numerous genes influencing a trait, ethnic differences and synonymous mutations for the genotype, to renal clearance, disease state, non-compliance, age and gender for the phenotype (Nebert et al, 2004). Increased feasibility of innovative technologies led genome-wide association studies to begin demonstrating that the genome is far more complex than had been previously appreciated.

Fifty years of DNA, from the 1953 double helix discovery to the 2003 Human Genome Project completion, have generated a number of answers and countless questions. Past its quindecennial, the human genome sequence, contracted from the predicted 100,000 genes to some 20,000 human protein-coding genes, remains a convoluted blueprint which has expanded knowledge beyond base pairs and transcription factors (International Human Genome Sequencing Consortium, 2004). The knowledge stems from the massive volumes of novel, and often entirely unanticipated elements which were uncovered and are proving constructive to human genetics, genomics and to pharmacogenetics.

1.1.2 The role of genes in psychiatric disorders

The philosophy of psychiatric disorders has been revolutionised and past ingenuous concepts precluded. Unworldly models of the cause, nature and neural substrates of mental disorders have been shelved together with the bygone beliefs that abnormal levels of one neurotransmitter or a single gene may systematically explain the pathogenesis of psychiatric disorders.

Compared to other illnesses, mental health care has long suffered from the narrow knowledge available on the biological basis of psychiatric disorders (Avramopoulos, 2010). In view of the genetic and polygenic complexity in the nature of mental illness, no recognised gene is either a prerequisite or sufficient to produce disease. A multitude of susceptibility genetic variants with small effects is involved, each augmenting the genetic disease risk by circa 1–2 percent (Bandelow et al, 2016). The combined involvement of genetic and environmental factors, which serve to explain a mental disorder, increases the complexity of the mental scenario.

Advancements have been made in gene expression studies, epigenetics and the identification of genes related to schizophrenia, such as *DISC1*, *TCF4*, *ZNF804A*, Alzheimer's Disease (*APOE*), attention deficit disorder (*DAT*, *DRD4*), bipolar affective disorder (*CACNA1C*, *ODZ4*), as well as *GABRA2* and *ADH4* for alcohol dependence, as reviewed by Nurnberger et al (2016). For complex diseases, the identification of susceptibility genes is approached through linkage studies that search for markers via a hypothesis-neutral methodology, association studies that investigate whether particular gene variants are more prevalent in patients than expected, or the assessment of intermediate phenotypes in that genetic polymorphisms may correlate to a cognitive symptom linked to more than one disorder. Schizophrenia, bipolar disorder and major depressive disorder, as an example, have been shown to have substantial shared genetic aetiology (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013).

Twin studies and family studies indicate that, to some extent, every psychiatric disorder studied has a genetic contribution. Variation at genetic level is relatively high for schizophrenia and autism whereas a heritability of around 40 percent is reported for anxiety and depression – making them as genetically determined as various other widespread conditions such as type 2 diabetes. For a number of complex psychiatric traits, a series of genetic loci implicated in disease mechanisms have been identified. Genetic analysis of depression persists among the greatest challenges facing researchers (Flint & Kendler, 2014).

The phenotypic heterogeneity of depression and the implicated distinct genetic architectures pose significant challenges. Imaging genetics in major depressive disorder, although at times not replicated, led to the first identification of risk-for-depression genetic variants. In comparison to non-carriers, the BDNF Val66Met 'Met' allele was

associated with higher volumes of gray matter and right middle frontal gyrus hyperactivation, while microstructural abnormalities, in white matter, predominantly the corpus callosum, were related to the 5-HTTLPR 'S' allele (Pereira et al, 2018).

An intriguing observation is that genetic makeup may moderate the response of an individual to stressful events in life. Caspi et al (2003) provided evidence of a gene-by-environment interaction through the study of functional polymorphisms in the serotonin transporter gene. Elevated depressive symptoms and suicidality related to stressful experiences were shown by individuals homozygous for the short allele of the 5-HTT gene, compared to heterozygotes or long allele homozygotes. In a study of major depression genetic architecture, 44 loci were identified through genome-wide association analyses, with the implication that all humans carry smaller or larger genetic risk factors for major depression (Wray et al, 2018).

1.1.3 Pharmacological management of depression

The role of brain derived neurotrophic factor, corticotropin-releasing factor, voltage activated Ca^{2+} channels, muscarinic acetylcholine receptors, the glucocorticoid receptor, and gene polymorphisms regulating the activity of ion channels in the neuronal membrane represent newer pathways, mechanisms and targets in the management of depression (Calker et al, 2018). Research on modifications in serotonergic and noradrenergic neurotransmission by antidepressant therapy has conversely presided the field for years, as evidenced by the main types of antidepressants available: tricyclic (TCAs) and related antidepressants, monoamine-oxidase inhibitors (MAOIs); selective serotonin re-uptake inhibitors (SSRIs); noradrenaline re-uptake inhibitors (NRIs); serotonin and

noradrenaline reuptake inhibitors (SNRIs); and noradrenergic and specific serotonin antidepressants (NaSSAs).

The 1950s marked the conception of tricyclic antidepressants (TCAs), with the introduction of imipramine (Kuhn, 1958). In the central nervous system (CNS), TCAs primarily inhibit the reuptake of noradrenaline and 5-hydoxytryptymine, with varying selectivity, as monoamine reuptake inhibitors (Mindham, 1982). The approval of amitriptyline by the Food and Drug Administration, in 1961, improved the management of agitated or anxious patients due to its more sedating action (Puri & Treasaden, 2011).

Sleep pattern improvement can be the foremost benefit of treatment with amitriptyline. Through the inhibition of the re-uptake of noradrenaline and serotonin by the presynaptic neuronal membrane in the CNS, amitriptyline increases the synaptic concentration of noradrenaline and serotonin. By way of constant receptor stimulation, chronic use of amitriptyline may produce downregulation of cerebral cortical β-adrenergic receptors and sensitisation of post-synaptic serotonegic receptors. The antidepressant effects are thought to result from an overall upsurge in serotonergic neurotransmission[1].

In attempting to explain the psychological effects sequence preceding recovery in patients responsive to amitriptyline, the study by Katz et al (1991) found that, within a week, amitriptyline acts on sleep disorder in all patients, and on anxiety and hostility in responders. Responders and non-responders become more distinguishable after 12 to 14 days of therapy, when steady state concentration of amitriptyline in plasma is correlatable to reductions in anxiety, hostility and somatic components of depression. Several weeks of treatment may be necessary in order to attain maximal clinical benefit with amitriptyline, as is the case with most antidepressants.

[1] DrugBank [Online]. Amitriptyline [accessed 2019 Apr 20]. Available from: https://www.drugbank.ca/drugs/DB00321.

Factors, specific to the patient, such as comorbid conditions and medications, or to the drug itself, such as dosing strategy, side-effects and cost, may modulate antidepressant selection. Tricyclic antidepressants, mirtazapine, bupropion, and venlafaxine are the preferred antidepressant options after SSRIs which, because of their safety profile, are generally considered first-line (Gautam et al, 2017). The National Institute for Health and Clinical Excellence (NICE) guidelines on depression in adults[2] highlight that there is weak evidence for the benefit of substituting within or between antidepressant classes. Amitriptyline appears to be equally tolerable to other antidepressants in terms of withdrawing treatment early, although patients taking other antidepressants tend to report fewer side effects. The NICE guidelines recommend considering the reintroduction of former medications that were ineffectively delivered or adhered to, including dose increases, and point towards a lower side-effect burden associated with antidepressant monotherapy compared to combination or augmentation therapy.

The Clinical Practice Guideline from the American College of Physicians concentrates on second-generation antidepressants as having lower toxicity in overdose than first-generation antidepressants (tricyclic antidepressants and monoamine oxidase inhibitors) and similar efficacy (Qaseem at al, 2016). The British Association for Psychopharmacology evidence-based guidelines suggest that tricyclic antidepressants (TCAs) should generally be reserved for cases when first-line treatment with SSRIs has failed and recommend the use of non-SRI antidepressants in patients with bleeding disorders and those taking aspirin or non-steroidal anti-inflammatory drugs. Reference is made in the literature to a marginal advantage in efficacy reported for amitriptyline, particularly in hospitalised patients whose status as inpatients may be tantamount to

[2] The British Psychological Society and the Royal College of Psychiatrists. The NICE guideline on the treatment and management of depression in adults: Updated Edition - Apr 2018 [Online]. National Clinical Practice Guideline 90 [accessed 2019 Apr 20]. Available from: https://www.nice.org.uk/guidance/cg90/evidence/full-guidline-pdf-4840934509.

higher severity and suicidality (Cleare et al, 2015). Although the difference in efficacy may be small overall, it may prove relevant when maximal response is required and in treatment-resistant patients.

Pharmacological resistance presents significant clinical challenges in the management of depression. Antidepressant therapy augmented with atypical antipsychotics, such as aripiprazole, may improve functioning (Weiller et al, 2018). Evidence is emerging in support of adjunctive nutraceuticals and novel approaches including ketamine and opioids. In tandem to the development of novel psychoactive medications, it is sensible to optimize the use of those presently available. Enhanced understanding of the pathophysiology of depression as a heterogeneous disorder and the corresponding variation in response and tolerability to treatment, may enable the revival of established drugs. Advances in genomics and cell biology provide an opportunity for rational design in targeting older therapies to improve efficacy and selectivity and therefore reducing toxicity (Ceskova & Silhan, 2018).

1.1.4 Efficacy and tolerability of antidepressants

The remarkable progress in central nervous system research still needs to translate into clinical benefit, especially in severe mental disorders where existing treatment is suboptimal. Thirty-five million dollars' worth of work on the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) clinical trial revealed that selective serotonin reuptake inhibitors (SSRIs) and newer-generation drugs are not essentially more efficacious than tricyclic antidepressants (TCAs) and older-generation drugs (Sinyor et al 2010, Cottingham et al, 2014).

Amitriptyline (as an example of the tricyclic antidepressants class) and fluoxetine are the only medicines used in depressive disorders listed in the 20th edition of the WHO Model List of Essential Medicines[3]. Amitriptyline, in particular, is also present in the list of medicines for pain and palliative care under the subheading 'Medicines for other common symptoms in palliative care'. Amitriptyline was not the first tricyclic antidepressant available and is not the best tolerated. It is, however, the reference drug against which efficacy and tolerability of antidepressants are evaluated (Barbui & Hotopf, 2001).

Amitriptyline, a tertiary amine tricyclic antidepressant (TCA), is more potent in inhibiting serotonin reuptake than secondary amine TCAs, such as nortriptyline. Amitriptyline is more potent in blocking the serotonin transporter, has greater α1–adrenergic receptor blockade and a high affinity for histamine H1 and muscarinic M1 receptors. Amitriptyline is the most anticholinergic antidepressant (Nelson, 2009). It is associated with weight gain, sexual dysfunction, orthostatic hypotension and QTc prolongation. Patients who have a clear clinical response upon initiating low-dose amitriptyline are maintained on such a dose with careful monitoring, recognising that risk of toxicity is high in overdose.

Jang et al (2009) attribute marked neurotrophic activity to amitriptyline which, in mice, has been demonstrated to act as an agonist of the TrkA and TrkB tyrosine kinase receptors, through which neurotrophins exert their physiological actions. The enhanced rigidity in the chemical structure of amitriptyline compared to other antidepressants, such as imipramine, is proposed to denote the potent and selective Trk agonistic activity of amitriptyline. Rantamäki et al (2011) suggest that transactivation of brain TrkB receptors

[3] World Health Organisation. WHO Model List of Essential Medicines: 20th Edition [Online]. WHO; Geneva: 2017 [accessed 2019 Apr 20]. Available from: http://www.who.int/medicines/publications/essentialmedicines/20th_EML2017_FINAL_amendedAug2017.pdf?ua=1.

is independent of monoamine reuptake blockade and brain derived neurotophic factor (BDNF). The central levels of BDNF, which plays an essential role in brain development and may be diminished in a number of neurodegenerative and metabolic diseases, such as the rare Wilms tumour-aniridia syndrome, may be increased by amitriptyline. In assessing the latter scenario, Daimon and colleagues (2013) highlight the potential pre-clinical therapeutic evidence emerging for amitriptyline in novel pharmacological disease contexts.

In the context of depression, evidence for amitriptyline endures, as is the case for a number of antidepressants, both new and old. In their auspicious review published in the Lancet, Cipriani et al (2018) accentuate that antidepressants do work - some are more tolerable and some are more effective than others. In the systematic review and network meta-analysis of 522 double-blind, randomised controlled trials comprising 116,477 adults with major depressive disorder, published until January 2016, all antidepressants were found to be more efficacious than placebo. Comparing 21 antidepressants in terms of the primary outcomes of efficacy (response rate) and acceptability (drop-out rate), agomelatine, amitriptyline, escitalopram, mirtazapine, paroxetine, venlafaxine, and vortioxetine were found to be more effective and agomelatine, citalopram, escitalopram, fluoxetine, sertraline, and vortioxetine were more tolerable than other antidepressants, with lower treatment discontinuation due to any cause. Some of the most widely prescribed antidepressants, such as fluoxetine and citalopram were among the least effective. Amitriptyline was reported to exert a far greater effect in managing depression symptoms with the odds ratio, of 2.13 (95% confidence interval 1.89 to 2.41), indicating that it was more than twice as likely to work as placebo. Amitriptyline was not the best tolerated but ranking first for efficacy, it may still be considered first choice for severe depression (Cipriani et al, 2018).

The review by Cipriani and colleagues makes no reference to the consideration of pharmacogenetics in relation to amitriptyline or any of the antidepressants studied to which this may be relevant, such as venlafaxine, sertraline, paroxetine, fluvoxamine, duloxetine, clomipramine, citalopram, and escitalopram. Amitriptyline was included in the study protocol of 82 trials, their respective results being disseminated between 1979 and 2003, with unclear or absent sponsorship declarations. The years '85, '88, '90 and '98, encompassed most of the research, with 6 publications each. It transpires that industry may have gradually stirred away from research in depression. Browsing through ClinicalTrials.gov[4], a global database of privately and publicly funded clinical studies, one retrieves hardly any studies conducted on the use of amitriptyline in depression during the past decade, with most of the recent research with amitriptyline focusing on migraine, fibromyalgia and pain, including myofacial, neck, abdominal, post-operative, chronic and neuropathic pain.

1.1.5 Tricyclic antidepressants for neuropathic pain

Neuropathic pain is 'caused by a lesion or disease of the somatosensory system' (Jensen et al, 2011), in contrast to the nociceptive origin of chronic pain arising from damage to non-neural tissue. Population prevalence of pain with neuropathic properties is estimated to be around 7-10 percent (van Hecke O et al, 2014), although indications point towards 20 percent of adults in Europe being potentially affected (Liedgens et al, 2016). Chronic pain, particularly back pain, often has both nociceptive and neuropathic components. The origin of neuropathic pain is complex with known causes including diabetic neuropathy,

[4] National Library of Medicine. ClinicalTrials.gov [Online]. National Institutes of Health; US [accessed 2018 Jul 7]. Available from: https://clinicaltrials.gov.

postherpetic neuralgia, amputations, trauma, and HIV infection. Notwithstanding the diversity in aetiologies, neuropathic pain is considered as a distinct clinical entity.

Patients with neuropathic pain are commonly diagnosed with comorbid depression. Studies indicate that depression affects 57 percent of individuals with pain and pain patients have 2 to 5 times higher risk for depression (Gureje et al, 1998, Jackson & St. Onge, 2003). TCAs, particularly tertiary amines, are the most effectively studied antidepressants for the treatment of neuropathic pain. Pain relief is achievable at lower doses that those entailed in the treatment of depression, and is believed to be independent of the antidepressant effects of these drugs. Multiple mechanisms are possibly involved, with theories ranging from the effect on serotonin and noradrenaline along descending spinal pain pathways, to the influence of TCAs on histamine receptors and the modulation of sodium channels (Sansone & Sansone, 2008).

Onali et al (2010) demonstrated that TCAs differentially regulate opioid receptors with direct stimulation of the δ or κ subtypes and suggest that this property may contribute to their analgesic activity. In neuropathic pain due to peripheral neuropathy, amitriptyline and nortriptyline were reported to be equivalent and if tolerated, a 23-26 percent visual analog scale pain reduction is expected. A discontinuation rate of 26-37 percent is anticipated due to inefficacy or adverse effects for either TCA (Liu et al, 2014). Amitriptyline has been studied in the treatment of fibromyalgia with results indicating that low doses of 10-75 mg per day are effective and amitriptyline should be considered a first-line drug in fibromyalgia (Rico-Villademoros et al, 2015). Amitriptyline acts at central and peripheral locations, modulating nociceptive and sensory processes at receptor and ion channel level. The multimodal mechanism of action, including

monoamine reuptake inhibition, is thought to cumulatively suppress the symptoms of fibromyalgia (Lawson, 2017).

Simple analgesics and non-steroidal anti-inflammatory drugs may be inadequate in alleviating persistent pain. Adjuvant therapy with antidepressants is currently practised in pain disorders with tricyclic antidepressants being effective in the management of neuropathic pain, low back pain, fibromyalgia and headaches. SSRIs exhibit limited and inconsistent analgesic effects but have a more favourable tolerability profile compared with tricyclic antidepressants (Dharmshaktu et al, 2012). Case study reports suggest that in painful neuropathy, 5% and 10% amitriptyline cream may have an analgesic dose-response effect (Kopsky & Hesselink, 2012). Topical treatment with tricyclics may improve patient compliance although systemic adverse reactions, such as drowsiness, may still manifest themselves.

Updated guidance from the Neuropathic Pain Special Interest Group of the International Association for the Study of Pain recommends TCAs, SNRIs, pregabalin, and gabapentin as first-line therapy. Data for lidocaine patches, opioids, cannabinoids and other combinations is weak or inconclusive. The systematic review and meta-analysis by Finnerup et al (2015) concluded that 16 out of the 18 placebo-controlled trials, evaluating amitriptyline in a daily dose of 25–150 mg, were positive. At the low doses prescribed for the control of pain, adverse effects, particularly sedation and dry mouth, resulting from the anti-muscarinic activity of amitriptyline, are still reported (Bryson & Wilde, 1996). Duloxetine and venlafaxine, being considered second-choice treatment in painful neuropathy, may be preferable to TCAs in patients with cardiovascular conditions and in the elderly (Janakiraman et al, 2016).

Albeit managed by 'unconventional analgesics' such as antidepressants and antiepileptics, treatment outcomes of neuropathic pain do not differ significantly from those of other chronic pain disorders. Merely a small proportion of patients achieve adequate response to therapy. As concluded by the 2015 Cochrane review on amitriptyline for neuropathic pain in adults, amitriptyline can be expected to give good relief to some patients while for others, it will not work (Moore et al, 2015). The explanation of this variation has yet to be fully understood.

1.1.6 Therapeutic drug monitoring for amitriptyline

A therapeutic window has been described for various tricyclic antidepressants but this gives no indication on whether the patient responds to the specific TCA, or not (Shimoda et al, 1997). Amitriptyline is readily absorbed in the gastro-intestinal tract, widely distributed throughout the body, metabolised in the liver and excreted in the urine. Systemic exposure is expected to be linear and predictable (Nam et al, 2015). The typical approach to initiating therapy has been to start at a low dose, increasing gradually until the therapeutic effect is attained or adverse effects become problematic. In view that alterations in dosage are not instantly translated into response or side effects, therapeutic drug monitoring (TDM) services were introduced by a number of laboratories to facilitate dosage adjustments (Dawling, 1988). In discussing the effectiveness of using therapeutic monitoring to guide TCA therapy, Dawling (1988) characterised a sound rationale based on (a) a relationship between the blood concentration and clinical outcomes; (b) a wide inter-individual variability in drug concentrations after standard dosing; (c) a low therapeutic index; (d) pharmacokinetics may be altered by disease or concomitant drug therapy; (e) toxicity may present severe risks to patients; (f) the clinical end-point may be

prolonged and not easily measured; (g) symptoms of inadequate dosing are not easily distinguished from those of drug toxicity.

Response, non-response and intolerability to tricyclic antidepressants may be linked to individual variations in pharmacokinetics and pharmacodynamics. Drug absorption, distribution, metabolism and excretion may vary depending on gender, age, morbidity, smoking or nutrition affecting the expression of drug targets or metabolising enzymes (Jefferson, 2011), whereas inter-individual differences in the expression or activity of receptors or transporters, which may be visualised by positron emission tomography or single photon emission tomography, represent pharmacodynamic peculiarities. Therapeutic monitoring, based on the supposition that clinical outcomes correlate better with blood levels than doses, is supported by the postulated correlation between amitriptyline plus nortriptyline concentrations in blood and the therapeutic and unwanted effects (Ostad Haji et al, 2012).

Therapeutic drug monitoring for amitriptyline is strongly recommended in the latest guidance for TDM in neuropsychopharmacology (Hiemke et al, 2018). In amitriptyline therapy, TDM is generally based on the determination of plasma or serum concentrations of amitriptyline and its main metabolite, nortriptyline. Burch et al (1981) reported the total amount of nortriptyline entering the systemic circulation is about one-quarter of the amitriptyline dose. For depression, a therapeutic reference range of 80 to 200 ng/mL is described for amitriptyline plus nortriptyline, while 300 ng/mL represents the alert level. A pharmacokinetic monitoring approach may be particularly relevant for amitriptyline in neuropathic pain management, since the lack of a widely accepted therapeutic reference range in the pain indication might render a pharmacodynamic approach less appropriate. The 2017 update of the Consensus Guidelines for Therapeutic Drug Monitoring in

Neuropsychopharmacology outlines how, irrespective of a therapeutic reference range, concentrations observed to be outside estimated dose-related reference ranges may enable identification of pharmacokinetic abnormalities which can influence systemic exposure to parent drug and metabolites (Hiemke et al, 2018). Blood levels outside the expected range may hint at disease-related changes, altered drug excretory functioning, and gene polymorphisms or drug-interactions that trigger aberrant metabolism. Irregular metabolism may not only influence the concentrations of amitriptyline and nortriptyline in blood, but also those of hydroxy-metabolites.

As illustrated in Figure 1-1, amitriptyline is metabolised mainly by demethylation in the hepatic cytochrome P450 system, forming nortriptyline, and by hydroxylation at the ethylene bridge of the central seven-membered ring to form isomeric alcohols, E- and Z-10-hydroxyamitriptyline. Nortriptyline is demethylated to the primary amine desmethylnortriptyline (NNT) and hydroxylated to E- and Z- 10-hydroxynortriptyline. Determination of the hydroxylated metabolites of amitriptyline and nortriptyline may prove beneficial given their potential correlation with significant clinical findings (Edelbroek et al, 1984, Young et al, 1984, Schneider et al, 1988, Shimoda et al, 1997, Steimer et al, 2005).

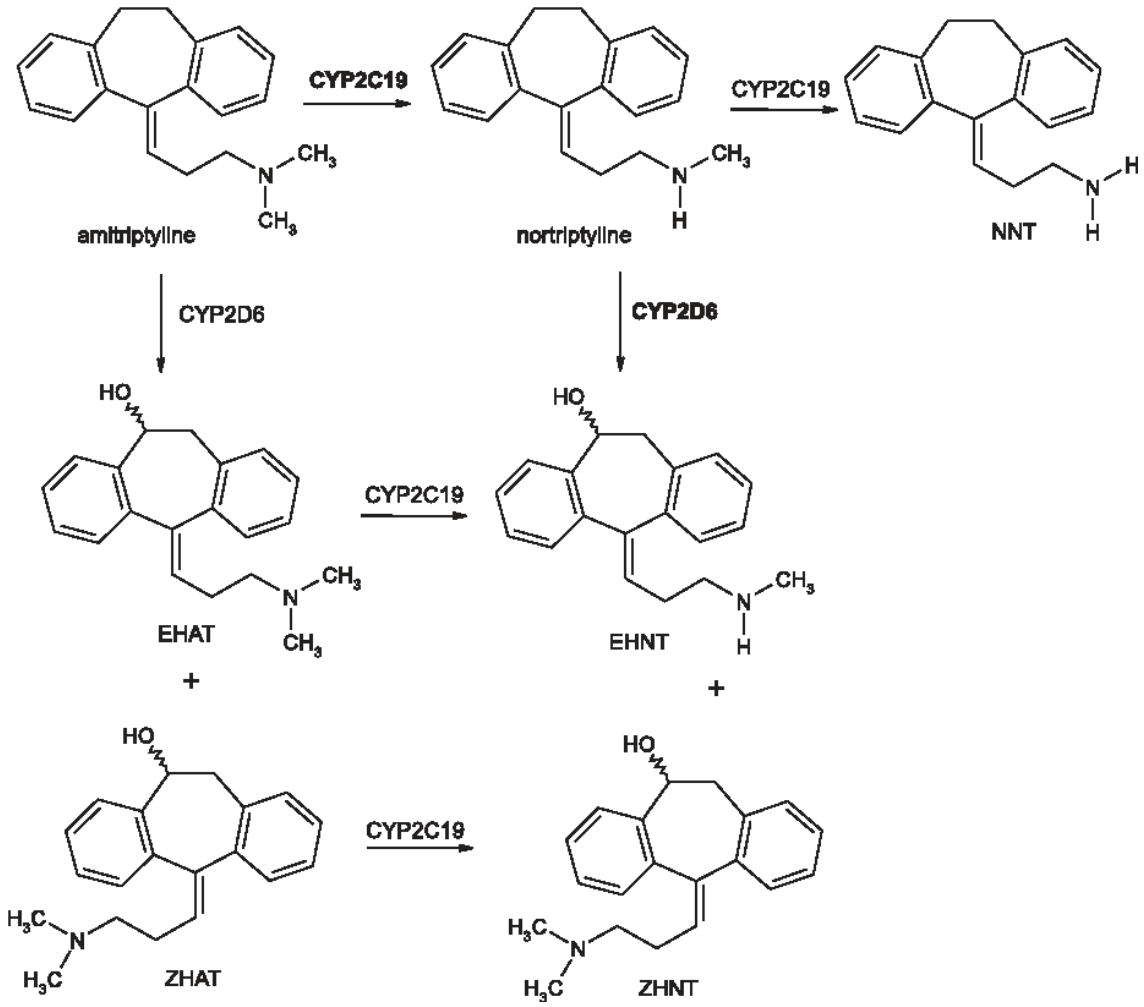
Progress in analytical techniques have made concentration measurements simpler but most procedures only account for the parent drug, and in the case of the tertiary amines, also for the active demethylated metabolite. Methods available for quantitative analysis of tricyclic antidepressants include immunoassay, high performance liquid chromatography (HPLC) and gas-liquid chromatography. Liquid chromatography tandem mass spectrometry, regarded as one of the most essential techniques of the last decade (Khatoon et al, 2013), has been employed for the quantification of amitriptyline

in human serum (Breaud et al, 2010), profiling of amitriptyline and some metabolites in human urine (Pesce et al, 2012, Chambers et al, 2014, Cao et al, 2015, Zhou et al, 2016) and plasma (de Castro et al, 2008), amitriptyline determination in oral fluid (Coulter et al, 2010) and other biological sources (Georgi & Boos, 2006, Chae et al, 2012). Levels of amitriptyline and nortriptyline in biofluids have also been studied through other systems, such as gas chromatography (Gupta et al, 1999).

Figure 1-1: Main metabolic pathways of amitriptyline
Major routes are shown in bold.

EHAT = E-10-hydroxyamitriptyline; ZHAT = Z-10-hydroxyamitriptyline; NNT = desmethylnortriptyline; EHNT = E-10-hydroxynortriptyline; ZHNT = Z-10-hydroxynortriptyline

Reproduced from Linden et al (2008), with permission from J Braz Chem Soc (Appendix A)



HPLC is the prevalent technique in published methods for the analysis of TCAs (Samanidou et al, 2008, Khatoon et al, 2013), with most reversed phase methods allowing simultaneous determination of tertiary and secondary amines. Chromatographic separation of tricylic compounds encompasses methods with column temperature varying from room temperature to 45 ˚C and detection of analytes being performed mainly using ultraviolet or diode array detectors covering a range of wavelengths (Linden et al, 2008). Capillary electrophoresis, a relatively new analytical technique, concedes low analysis time albeit low sensitivity.

Shimoda et al (1997) employed an HPLC system with an absorbance detector at a wavelength of 200 nm for examining levels of amitriptyline and metabolites in plasma, as predictors of the clinical antidepressant outcome. They report that increasing concentrations of amitriptyline and cis-isomers of hydroxylated metabolites relate to a better clinical sequel, while increasing concentrations of nortriptyline and trans-isomers of hydroxylated metabolites predict a poor outcome in depressive episodes. The 10-OH metabolites of amitriptyline and nortriptyline possess approximately half the potency of their parent compounds, with the E-10-hydroxynortriptyline concentration having a strong negative correlation with percentage improvement. Marked individual variation is recognised, presumably caused by differences in liver enzyme activity. The lack of correlation between amitriptyline or nortriptyline concentrations and E-10-hydroxynortriptyline, points towards the potential worth of considering E-10-hydroxynortriptyline in establishing the therapeutic concentration range (Edelbroek et al, 1984), mindful that hydroxy-metabolites of nortriptyline have been associated with cardiovascular toxicity in early studies reporting high plasma levels following intake of therapeutic doses (Schneider et al, 1988, Young et al, 1984).

Upward et al (1988) report ECG changes which include shortened sinus cycle length with doses of 150-200 mg amitriptyline daily, together with an 8% and 10% prolongation of the PR interval and QRS duration, respectively. Amitriptyline features on the long list of drugs that cause QT prolongation and has been implicated with the specific form of ventricular tachycardia known as torsades de pointes. TCAs prolong the QTc predominantly by blocking the Na^+ channel (Nachimuthu et al, 2012). The effect is more pronounced by the inhibition of outward K^+ channels (Lionte et al, 2012). ECG changes are most evident in overdosage. It is suggested that tricyclic antidepressants may unmask subclinical dysfunctional sodium channels and trigger drug-induced sudden death in patients receiving chronic treatment (Yap & Camm, 2003). Interestingly, Maslej et al (2017) support the hypothesis that antidepressants are less harmful in cardiovascular patients than in the general population. Their meta-analysis describes how antidepressant use was linked to increased risks of mortality and new cardiovascular events in general-population samples but in cardiovascular patients, risks were not significantly increased by antidepressant use.

1.1.7 Genetic polymorphisms concerning amitriptyline metabolism

Population pharmacokinetics and interpatient variability may delineate the variable outcomes. Systemic exposure to amitriptyline and its metabolites within an individual patient is influenced by CYP2C19 and CYP2D6, cytochrome P450 subfamily enzymes, known to be subject to genetic polymorphism. CYP2C19 primarily catalyzes the demethylation of amitriptyline and nortriptyline, with CYP1A2, CYP2C9, and CYP3A4 possibly participating at higher drug concentrations (Venkatakrishnan et al, 1998). CYP2D6 is stereospecific for the (-)-E-10-hydroxyamitriptyline and (-)-E-10-hydroxynortriptyline metabolites and responsible for the formation of the E-10 hydroxy-

metabolites (Breyer-Pfaff, 2004). A varied spectrum of enzymatic activities arises from the highly polymorphic nature of *CYP2C19* and *CYP2D6*, ranging from absent to normal or even increased activity. Linden et al (2008) propose evaluation of CYP2C19 activity through the evaluation of demethylation metabolic ratios after a single oral amitriptyline dose. CYP2D6 presents a more complicated scenario marked by more than 100 variant alleles, a range of single nucleotide polymorphisms (SNPs), pseudogenes, hybrid crossovers, gene conversions, short insertion and deletions, and copy number variations (CNVs), comprising gene deletion and multiplications of whole gene (Langaee et al, 2015). Scantamburlo et al (2017) recommend the utilisation of two separate methods for intra-patient validation of genetic variation particularly in view of potential allele dropout, which may cause alleles to remain undetected due to interference by other SNPs.

A range of chemistries have been technologically advanced for genotyping *CYP2C19* and *CYP2D6*. DNA sequencing is the gold standard against which genotyping platforms, ranging from in-vitro diagnostic (IVD) tests to laboratory-developed tests, are compared (Black, 2014). These include probe-based methods such as TaqMan® assays (Life Technologies), high-resolution melting (HRM), bead chip (Luminex) and DNA chip (AmpliChip CYP450 kit). In 2005, Roche Molecular Systems (Branchburg, NJ) was granted market clearance for AmpliChip CYP450 Test, through the Office of In-Vitro Diagnostic Device Evaluation and Safety of the FDA. This first pharmacogenetic test, using a DNA microarray for genotyping the *CYP2D6* and *CYP2C19* genes involved in the metabolism of many antipsychotics and antidepressants, delivered pharmacogenetic testing to psychiatry (de Leon et al, 2009).

Genotyping techniques commonly rely on direct detection or adopt a secondary polymerase chain reaction (PCR) in their approach. Direct detection methodologies in

which specificity to the intended target is ensured by the primers in a single PCR assay, are generally faster. Commercial DNA chip/bead based methods frequently apply the secondary approach where the region of interest is selectively amplified by a primary PCR followed by the actual genotyping assay. This entails higher workload and cost but allows discrimination between more alleles with less bias (Larsen & Rasmussen, 2017).

A combination of specific SNPs and interrogated sequence variants define the haplotype which is designated a star-allele (*) nomenclature for standardisation. Alleles are allocated an activity value. Laboratories typically report a diplotype, denoting the inherited maternal and paternal star-alleles (Hicks et al, Suppl 2017). The activity values of each of the alleles reported in the diplotype are summed to determine an activity score. For instance, the *CYP2D6*1/*17* diplotype activity score is 1.5: the activity value of normal function **1* (=1) added to the activity value of decreased function **17* (=0.5). In attempting interpretation of genotyping results, two dysfunctional alleles are predicted to denote a poor metaboliser, heterozygous carriers are termed intermediate metabolisers, carriers of two wild-type normal function alleles are classified as extensive metabolisers, while duplicated active alleles may define ultra-rapid metabolisers.

Analysis of copy number variations has been marked by the inability of conventional methods to determine which of the two *CYP2D6* alleles in a sample carries duplication or multiplication. This may be relevant in the process of inferring in-vivo enzyme activity in that CNVs are observed for the normal, non-functional and also reduced activity *CYP2D6* alleles. Techniques have evolved from long-range and multiplex PCR, TaqMan® real-time PCR, restriction fragment length polymorphism, to microarray hybridization-based methods and allele quantification-based pyrosequencing genotyping (Langaee et al, 2015). Two copies (N=2) of *CYP2D6* normal alleles (e.g. **1×N*, **2×N*)

have been attested to enhance CYP2D6 enzyme activity with the frequency of duplication existing in around 1% of Caucasian and Asian populations, and 1.6-3.3% of African populations. Deletion of *CYP2D6*, referred to as C*YP2D6*5*, occurs similarly in Caucasian, Asian and African populations (2–7%). The impact on inferring genotype-derived phenotypes cannot be ignored (He et al, 2011).

Allele frequencies at large have been shown to vary considerably among world populations. Some variations are observed at comparable frequencies across populations, while others are present in different frequencies or may have only been reported in a particular ethnicity (Gaedigk et al, 2017). Table 1-1 provides examples of allelic variants and function, with corresponding frequency in European and North American populations.

Table 1-1: *CYP2C19* and *CYP2D6* variants

Gene	**Function**	**Alleles**	**Caucasian frequency**	**Gene**	**Function**	**Alleles**	**Caucasian frequency**
CYP2D6	Normal	**1*	37.123	*CYP2C19*	Normal	**1*	0.624
		**2*	26.833				
		**33*	1.900				
		**34*	3.767				
		**35*	5.300				
		**39*	7.092				
	Increased: *CNV* multiplication	**1xN*	0.991		Increased	**17*	0.213
		**2xN*	1.119				
		**35xN*	0.208				
	None	**3*	1.364		None	**2*	0.146
		**4*	18.174				
		**5*	2.829				
		**7*	0.094				
		**13*	0.187				
		**31*	0.095				
		**56*	0.653				
	Decreased	**10*	2.780				
		**17*	0.312				
		**29*	0.142				
		**41*	8.699				
		**59*	0.650				

Alleles with unclear function and/or frequency <0.05 were excluded.

Adapted from the Pharmacogene Variation Central Repository, PharmVar (https://www.pharmvar.org/genes) and the Pharmacogenomics Knowledgebase, PharmGKB (https://www.pharmgkb.org).

The implications of genetic polymorphisms are further complicated by drug-drug-gene interactions occurring when another drug in the patient's regimen affects the individual's enzyme activity pertinent to amitriptyline. Phenocopying during TCA use may be precipitated by concurrent prescription of SSRIs which cause drug-induced poor metaboliser phenotypes. Paroxetine and fluoxetine are potent CYP2D6 inhibitors while CYP2C19 may be inhibited by fluoxetine and fluvoxamine. Data indicates that around 20 percent of patients receiving treatment for depression may convert to CYP2D6 poor metaboliser status (Preskorn et al, 2013) and thus, those taking potent inhibitors of CYP2D6 should be treated similarly to CYP2D6 poor metabolisers (Crews et al, 2012).

The additional concern of physiologically based pharmacokinetic interactions is highlighted by Loan and colleagues (2012) that point towards a change in the kinetic behaviour of amitriptyline by amlodipine-induced alterations in blood pressure. The heterogeneity of disorders for which amitriptyline is prescribed, the confounding rates of spontaneous remission and placebo response and the sub-therapeutic concentrations resulting from pervasive poor compliance, intensify the challenges in clinical practice (Mitchell, 2000).

1.1.8 Clinical relevance of pharmacogenetics in amitriptyline therapy

Owing to the long biological half-lives of these drugs, around 24 hours for amitriptyline (Gupta et al, 1999), optimization of therapy by dose adjustments in response to blood concentrations or adverse events, may be prolonged. Early discontinuation of antidepressant therapy due to adverse reactions or lack of improvement is common, occurring in about 30 percent of patients by week six (Peñas-Lledó et al, 2013). Considering inter-individual variability in drug metabolism, starting with low doses as is

standard practice, may enhance tolerability in CYP2D6 poor metabolisers whereas ultra-rapid metabolisers may present a higher discontinuation rate, owing to the diminished effect. Genotyping before amitriptyline therapy may enable identification of discontinuation risk.

Ryu et al (2017) demonstrated the dependence of amitriptyline pharmacokinetic parameters on both CYP2C19 and CYP2D6. CYP2C19-mediated N-demethylation, rather than CYP2D6-mediated hydroxylation, was identified as the dominant metabolic pathway for amitriptyline, with *CYP2C19* non-functional alleles denoting higher systemic exposure to amitriptyline. Earlier in 2005, Steimer et al, showed that there is a significant correlation between nortriptyline, but not amitriptyline concentrations, and adverse events. This enthused the notion that, contrary to the CYP2D6 scenario, slow CYP2C19 metabolisers may experience less adverse effects than fast metabolisers. Co-therapy with low doses of a CYP2C19 inhibitor, perhaps one with potential synergistic effects (e.g. SSRIs) may in turn allow administration of effective doses without intolerable adverse effects, particularly in CYP2C19 intermediate metabolisers.

Jornil & Linnet (2009) argue that a CYP2C19 or CYP2D6 poor metaboliser status is unlikely to trigger serious amitriptyline intoxication, while combined reduced levels of other CYP isoenzymes or drug-induced inhibition of metabolism may be contributing factors associated with amitriptyline poisoning. Patients suffering from depression and pain syndromes may be on one or more antidepressants and other serotonergic drugs (e.g. tramadol) with the conceivable interactions intensifying the clinician's struggle to appreciate the practicality of prescribing TCAs. Commercial influences may have driven the overstatement of risks and disadvantages of the tricyclics, compared to newer drugs

(Gillman, 2007). As new knowledge emerges and technologies become available, the need of updating pharmacological data concerning TCAs can be addressed.

Clinicians need direction in interpreting novel information. The availability of guidelines translating pharmacogenetic test results into clinical decisions for individual patients is a recognised necessity (Swen et al, 2018). The Clinical Pharmacogenetics Implementation Consortium (CPIC), established as a joint effort between PharmGKB and the Pharmacogenomics Research Network in 2009, publishes guidelines designed to assist clinicians in understanding how genetic test results may be utilised to optimise drug therapy. The Pharmacogenomics (PGx) Working Group of the Association for Molecular Pathology Clinical Practice Committee supports CPIC by defining recommendations for a 'must-test' list in attempt of standardising the variants tested. The work started with *CYP2C19* for which a 2-tier system was proposed. The set in tier 1 - minimum panel of variant alleles, includes **2*, **3*, and **17* whereas other variants were included in tier 2 - extended panel of variant alleles (Pratt et al, 2018).

The 2012 Clinical Pharmacogenetics Implementation Consortium Guideline for *CYP2D6* and *CYP2C19* Genotypes and Dosing of Tricyclic Antidepressants (Hicks et al, 2013) was followed by the 2016 update (Hicks et al, 2017). The latest guideline and its supplement provide an array of resources including tables for *CYP2D6* and *CYP2C19* alleles definition, functionality, frequency, activity scoring system to quantitate the predicted functional status of CYP2C19 and CYP2D6, diplotype-phenotype interpretation, as well as clinical decision support for amitriptyline. CYP2C19 impacts the conversion of parent tertiary amine to secondary amine, but may have less influence than CYP2D6 on overall drug clearance. Specific combinations of *CYP2D6*

and *CYP2C19* alleles, as described in Table 1-2, are likely to result in additive effects on the pharmacokinetic properties of amitriptyline.

Table 1-2: Clinical Pharmacogenetics Implementation Consortium (CPIC) dosing recommendation for amitriptyline based on CYP2D6 and CYP2C19 phenotypes

Recommendations to be interpreted in line with the *Clinical Pharmacogenetics Implementation Consortium Guideline for CYP2D6 and CYP2C19 Genotypes and Dosing of Tricyclic Antidepressants* (adapted from Hicks et al, 2017)		**Phenotype**	*CYP2D6 ultra-rapid metaboliser*	*CYP2D6 normal metaboliser*	*CYP2D6 intermediate metaboliser*	*CYP2D6 poor metaboliser*
		Genotype	duplications of functional alleles	combinations of alleles that result in an activity score of 1.0–2.0†	1 decreased function and 1 no function allele†	only no function alleles
Phenotype	**Genotype**	**Frequency**	*~1–20%*	*~72–88%*	*~1–13%*	*~1–10%*
CYP2C19 ultra-rapid or rapid metaboliser	2 increased function alleles; or 1 normal function allele and 1 increased function allele	*~2–5%; or ~2–30%*	Avoid amitriptyline use	Consider alternative drug not metabolised by CYP2C19	Consider alternative drug not metabolised by CYP2C19	Avoid amitriptyline use
CYP2C19 normal metaboliser	2 normal function alleles	*~35–50%*	Avoid amitriptyline use; if amitriptyline is warranted, consider titrating to a higher target dose	Initiate therapy with recommended starting dose	Consider 25% reduction of recommended starting dose	Avoid amitriptyline use; if amitriptyline is warranted, consider 50% reduction of recommended starting dose
CYP2C19 intermediate metaboliser	1 normal function allele and 1 no function allele or 1 no function allele and 1 increased function allele	*~18–45%*	Avoid amitriptyline use	Initiate therapy with recommended starting dose	Consider 25% reduction of recommended starting dose	Avoid amitriptyline use; if amitriptyline is warranted, consider 50% reduction of starting dose
CYP2C19 poor metaboliser	2 no function alleles	*~2–15%*	Avoid amitriptyline use	Avoid amitriptyline use; if amitriptyline is warranted, consider 50% reduction of starting dose	Avoid amitriptyline use	Avoid amitriptyline use

† Assigning activity score of 2.25 as CYP2D6 normal metaboliser and downgrading an activity score of 1 to the CYP2D6 intermediate metaboliser group is recommended in the Consensus CYP2D6 genotype to phenotype table - March 2019

Two *CYP2C19* normal function alleles and at least one *CYP2D6* no function allele increase risk of side effects with amitriptyline, while patients having two *CYP2D6* normal function alleles with at least one *CYP2C19* no function allele have a lower risk of experiencing side effects. Limited data is available on dose adjustments based on the combinatorial CYP2C19 and CYP2D6 metaboliser phenotype (Hicks et al, 2017). In the CPIC Guideline, the CYP2D6 predicted phenotype is assigned as 'poor metaboliser' for an activity score of 0, 'intermediate metaboliser' for an activity score of 0.5, 'normal metaboliser' for activity scores between 1.0 and 2.0, and 'ultra-rapid metaboliser' for activity scores greater than 2.0. For instance, a pharmacogenetic test result of *CYP2D6*1/*17* with an activity score of 1.5 predicts a normal metaboliser phenotype.

The CPIC CYP2D6 Phenotype Standardization Project sought to standardize phenotype prediction from genotype data since CYP2D6 phenotype reports, based on genotyping, are not consistent across laboratories. The March 2019 Consensus[5] presents the rationales for downgrading a *CYP2D6* activity score of 1.0 from 'normal' to 'intermediate' metaboliser; for downgrading *CYP2D6*10* activity score from 0.5 to 0.25; and for assigning an activity score of 2.25 as CYP2D6 normal metaboliser. The newly proposed measures, including a continuous scale for activity score, may denote an update to reported genotype-inferred phenotypes and corresponding clinical recommendations.

Drug clearance altered by CYP2D6 or the parent-to-metabolite ratio being shifted by CYP2C19 may predispose patients to treatment failure or side effects. While amitriptyline is associated with anticholinergic, central nervous system and cardiac effects, the CYP2C19 metabolite, nortriptyline, is mainly linked to anticholinergic effects and cardiotoxicity, whereas the CYP2D6 hydroxy-metabolites may cause arrhythmias,

[5] Clinical Pharmacogenetics Implementation Consortium. CYP2D6 Genotype to Phenotype Standardization Project [Online]. CPIC; US: 2019 [accessed 2019 Jul 7]. Available from: https://cpicpgx.org/resources.

heart block and tachycardia (Hicks et al, Suppl 2017). In view of the lower amitriptyline doses used for neuropathic pain treatment, and the limited data available in this setting, CPIC recommend a cautious approach for TCAs in pain management. CYP2D6 ultra-rapid metabolisers may be at higher risk of failing therapy due to amitriptyline concentrations being less than expected. Close monitoring is suggested for patients having a combination of poor or ultra-rapid phenotypes for CYP2D6 and CYP2C19.

The Dutch Pharmacogenetics Working Group (DPWG), established by the Royal Dutch Pharmacists Association in 2005, also provides PGx-based therapeutic recommendations for known gene-drug pairs (Bank et al, 2018). In the Netherlands, DPWG guidance is integrated in the national drug database which incorporates decision support information in electronic prescribing and pharmacy systems. If a patient's genotype is available in the automated medication surveillance system, an alert pops up upon prescription of a medicine with a pharmacogenetic guideline for this gene (Cheung et al, 2017). A similar approach is adopted at Mayo Clinic, through the Pharmacogenomics Task Force, whereby clinicians are presented with updated patient genotype information and decision support guidelines recommend adjustments in dose or substitution to a drug with a different metabolic pathway. Nassan et al (2016) explain that the advantages of such recommendations, applied alongside clinical judgement offset the risks and although the evidence base of pharmacogenetics in antidepressant therapy may not be as robust as, for instance, targeted medication in oncology, it might not be in the patients' best interest to wait for such robust evidence while depriving them of safer therapy.

The French national network of pharmacogenetics (RNPGx) provides three levels of recommendation for pharmacogenetic testing: 'essential', where impact on clinical phenotype is of major importance and difficult to predict by non-genetic approaches;

'advisable', where there is demonstrated functionality as a complement to phenotyping or to predict drug exposure for therapeutic management and 'possibly helpful' where functionality is probable and may be applied on a case-by-case basis depending on the clinical context (Picard et al, 2017). Caraballo et al (2017) propose an operational model to facilitate implementation of pharmacogenomics (PGx) in routine prescribing which is based on eight functional components, namely: (i) institutional leadership support including resources; (ii) pharmacogenomics governance in a multidisciplinary team; (iii) PGx education and promotion; (iv) PGx knowledge and its management; (v) clinical approval and assessment of practice impact; (vi) laboratory results standardisation and storage; (vii) clinical decision support (CDS) integrated in the electronic health record (EHR); (viii) long-term maintenance encompassing regular updates. Borobia and colleagues (2018) describe the feasibility of implementing pharmacogenetic testing within a university hospital supported by the Spanish national health system. The established pharmacogenetics unit, aimed at pre-emptive genotyping in risk populations and individualisation of clinical recommendations, received over 2500 consultation requests within the first 3 years (Borobia et al, 2018).

Research on the clinical implications of pharmacogenomics is a priority in the European Union with considerable funds being allocated to projects like GENDEP (Genome-based therapeutic drugs for depression) which focused on escitalopram and nortiptyline (Hodgson, 2014), and the ongoing project of Ubiquitous Pharmacogenomics (U-PGx) which aims to increase accessibility to effective treatment optimisation. The clinical study PREPARE[6] - Preemptive Pharmacogenomic Testing for Preventing Adverse Drug

[6] Ubiquitous Pharmacogenomics Consortium. PREPARE: Making effective treatment optimization accessible to every European citizen [Online]. U-PGx [accessed 2019 Jul 13]. Available from: http://upgx.eu/study/.

Reactions - initiated by the U-PGx consortium, has recruited over 5000 patients in seven European clinical centres, with the goal of disseminating initial outcomes by 2020.

The heterogeneity of clinical outcomes is impacted by the effect of genetic variants on both pharmacokinetic and pharmacodynamic drug parameters. Pharmacodynamic parameters, which may be more significant for certain drugs, as in oncology, represent a challenge which goes beyond linking genetic factors to drug concentrations, and fewer examples are available to form the basis for guidance (Maliepaard et al, 2013). Data and experience is relatively mature for the genetic influence on pharmacokinetic properties, particularly in relation to drug metabolism. In critical dose drugs, genotypic identification of individuals with slightly reduced metabolism may be more significant than expected, and could be strategic in demonstrating genotyping cost-effectiveness, beyond the mere screening for extremes in the metabolism continuum (Steimer et al, 2005).

1.1.9 Regulatory sciences perspective

Regulators are confronted by the challenge of ensuring that the practices proposed to remodel the conventional phenotype-based approach of pharmacological treatment, are appropriate and fit for purpose (Prasad & Breckenridge, 2011). Drug labels may contain pharmacogenomic data available at time of the initial registration. In a number of known examples, it was only after registration of the medicinal product that the consequences of genetic polymorphism were recognised[7]. Pharmacogenetic research conducted after the regulatory approval of particular drugs, such as warfarin, codeine and clopidogrel, yielded information that helps optimize their risk-benefit profile and has been included in drug

[7] European Medicines Agency. Guideline on good pharmacogenomic practice – Draft [Online]. EMA; 2016 [accessed 2019 Apr 22]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/05/WC500205758.pdf.

labelling. Genomically-guided research is ideally carried out for all medications used commonly in clinical practice, in attempt of improving efficacy, limit serious side-effects and promote cost effectiveness of currently marketed drugs. Pharmaceutical companies, cognisant that precision medicine could possibly compromise their drugs' market share, may have no business incentive to advocate this strategy (Harper & Topol, 2012). Studies on older drugs such as tricyclic antidepressants are difficult to perform, one reason being that these drugs may no longer be recommended as first-line therapy. Direct involvement of industry to drive the application of pharmacogenomics to older products, where public health gain could be expected, is not likely, with public-private endeavours such as the Innovative Medicines Initiative, being anticipated to support studies investigating marketed drugs (UK Pharmacogenetics Study Group, 2006).

The progress of pharmacogenomics into the clinic setting is likely to be affected by differences in legislation between regions whereby ethical principles may differ along with the legal requirements for collection and storage of samples and data. The rapid scientific development in the field of pharmacogenomics imparts an important role on regulators to achieve consistency in interpretation, with the foremost issue being clear definitions of the reference terminology (Prasad, 2009). Guidelines issued by the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, with regulatory implementation approved by the European Medicines Agency (EMA), adopted by the Japanese Ministry of Health, Labour and Welfare and published in the Federal Register by the FDA, facilitate harmonisation. ICH topic E15, entitled *Definitions for Genomic Biomarkers, Pharmacogenomics, Pharmacogenetics, Genomic Data and Sample Coding Categories,* provides guidance on definitions established between regulators, academia and industry collaborating in a unified framework for scientific discussion (Maliepaard et al, 2013).

This route established agreement on the application of consistent terminology in the discipline of pharmacogenomics and pharmacogenetics in all ICH constituents (ICH Harmonised Tripartite Guideline E15, 2007). A common language among sponsor companies, ethics committees, research participants and the regulator provided the foundation for subsequent regulatory documents and successful dialogue at individual regulatory authority, regional and ICH levels.

Regulatory agencies in different ICH regions review submissions by industry proposing the incorporation of pharmacogenomic biomarkers for specific purposes. The provision of harmonised recommendations[8] complements the simultaneous development and regulatory appraisal of the use of genomic biomarkers by outlining the qualification context and intended use claims, determining standard data collection methods, and establishing formats for data submission to regulatory authorities, assisting efficient reviews. ICH Topic E16, *Genomic Biomarkers Related to Drug Response: Context, Structure and Format of Qualification Submissions*, recommends simultaneous qualification submissions to pertinent regulatory authorities, to facilitate the integration of biomarkers in the development of drugs and biotechnology products globally (ICH Harmonised Tripartite Guideline E16, 2010).

In June 2014, the ICH Steering Committee endorsed the topic *Genomic Sampling and Management of Genomic Data* (E18)[9] with the objective of integrating the experiences of both regulatory authorities and pharmaceutical industries in the context of appropriate planning for standardised unbiased genomic sample collection, coding, and storage, for

[8] ICH. Genomic Biomarkers Related to Drug Response: Context, Structure and Format of Qualification Submissions. ICH Final Concept Paper E16 [Online]. ICH; 2008 [accessed 2019 Jul 28]. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E16/Concept_papers/E16_Concept_Paper.pdf.

[9] ICH. Genomic Sampling and Management of Genomic Data. ICH Final Concept Paper E18 [Online]. ICH; 2014 [accessed 2019 Jul 28]. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E18/E18EWG_ConceptPaper_Final2014_0605.pdf.

both pre-specified and future use through retrospective analysis. Harmonisation across regions shall maximise the information gathered from sample collection and analysis while considering transparency of findings in line with local legislation, so as to facilitate the implementation of pharmacogenomics for the benefit of all stakeholders (ICH Harmonised Guideline E18, 2017).

Harmonisation does not happen overnight. Progressive change has ensued in European pharmaceutical legislation since the Directive of 1965 (Directive 65/65/EEC), which was intended to harmonise medicines approval standards within the then European Economic Community. In 1975, the Committee for Proprietary Medicinal Products (CPMP) was established as the main advisory committee to the European Commission (Directive 75/319/EEC). The mutual recognition of approved products in member states represented the first effort at harmonisation. In 1993, the role of the European Medicines Agency (EMA, formerly EMEA) was established (EEC/2309/93) to facilitate a centralized authorization across all member states for certain medicinal products, and the council regulation on human medicines EC 726/2004, included the decentralized procedure and established the Coordination Group for Mutual Recognition and Decentralized Procedures (human), CMD(h), to harmonise further the drug regulatory process (Prasad & Breckenridge, 2011). The national procedure, via single national competent authorities, was conserved for medicines authorised before EMA creation and those not in the scope of the centralised procedure.

The Scientific Advice Working Party (SAWP), established in May 2004, provides a formal process for sponsors and developers to seek advice. The Committee for Medicinal Products for Human Use (CHMP), which replaced CPMP as of 2004 upon broadening of its responsibilities, established a multidisciplinary expert group, formalised in 2005 as the

Pharmacogenomics Working Party. It is composed of fourteen experts nominated by CHMP, based on their regulatory experience and scientific expertise, and selected experts from a European list maintained by the EMA. The Pharmacogenomics Working Party (PgWP) offers informal opportunities and a common forum for discussion which may involve the Food and Drug Administration (FDA) and the Pharmaceuticals and Medical Devices Agency (PMDA) in Japan. The Pharmacogenomics Working Party provides recommendations to the CHMP, on general and product-specific matters relating to pharmacogenomics, supports dossier evaluation and develops guidelines for the preparation and evaluation of the pharmacogenomic parts of the regulatory submissions. In liaison with other working parties, PgWP presents a technical forum to applicants and to the network, catalysing the integration of pharmacogenomics in drug development, assessment and information[10]. Furthermore, in line with the 2012 pharmacovigilance legislation, the EMA established the Pharmacovigilance Risk Assessment Committee (PRAC), to strengthen the monitoring of safety of medicines across Europe.

Initiatives such as the Innovation Task Force, think tank meetings and collaborative efforts embody EMA's endeavours to revisit the drug and biomarker development approaches and enhance the level of regulatory support and involvement. As outlined in Table 1-3, the European Medicines Agency, over the years, issued reflection and concept position papers for consultation and adopted scientific guidelines on pharmacogenomics.

[10] European Medicines Agency. Mandate, objectives and rules of procedure for the CHMP PG working party [Online]. EMEA; 2009 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Other/2010/01/WC500069715.pdf.

Table 1-3: Guidance documents issued by the European Medicines Agency in relation to pharmacogenomics

Technical pharmacogenomic aspects
EMEA/CPMP/3070/2001 Position paper on terminology in pharmacogenetics
EMEA/CHMP/PGxWP/201914/2006 Reflection paper on pharmacogenomic samples, testing and data handling
EMA/CHMP/PGWP/415990/2014 Concept paper on good genomics biomarker practices
EMA/CHMP/718998/2016 Guideline on good pharmacogenomic practice
Pharmacogenomic considerations during drug life cycle
EMA/CHMP/641298/2008 Reflection paper on co-development of pharmacogenomic biomarkers and assays in the context of drug development
EMA/CHMP/37646/2009 Guideline on the use of pharmacogenetic methodologies in the pharmacokinetic evaluation of medicinal products
EMA/CHMP/446337/2011 Reflection paper on methodological issues associated with pharmacogenomic biomarkers in relation to clinical development and patient selection
EMA/CHMP/281371/2013 Guideline on key aspects for the use of pharmacogenomics in the pharmacovigilance of medicinal products
EMA/CHMP/644998/2016 Concept paper on an addendum on terms and concepts of pharmacogenomic features related to metabolism to the guideline on the use of pharmacogenetic methodologies in the pharmacokinetic evaluation of medicinal products
Therapeutic-specific pharmacogenomics
EMEA/CHMP/PGxWP/128435/2006 Reflection paper on pharmacogenomics in oncology
EMEA/CHMP/PGxWP/278789/2006 Reflection paper on the use of genomics in cardiovascular clinical intervention trials
Procedural
EMEA/CHMP/PGxWP/20227/2004 Guideline on pharmacogenetics briefing meetings
Joint EMA-FDA VGDS /2006 Processing joint Food and Drug Administration and European Medicines Agency voluntary genomic data submissions within the framework of the confidentiality arrangement

In Europe, the legislation that governs devices is distinct from pharmaceutical legislation and the EMA remit is limited to medicines regulation. The in vitro diagnostics (IVD) directive first introduced in 1998, IVD Directive (98/79/EC), provides for the Conformité Européen (CE) marking for diagnostic devices. Clinical validation requirements prior to

CE marking and authorization for companion diagnostics, classed as IVDs, may vary from those entailed for pharmaceuticals. The new In Vitro Diagnostic Device Regulation (EU) 2017/746, published in May 2017, will come into full force in 2022, intending to strengthen the approval system through a risk-rule classification system which takes patient impact into consideration. Despite the changes, the European Union is still retaining separate pathways for companion diagnostics (CDx) and drugs even though splitting of regulatory responsibilities may hinder co-development of a medicine and its performance-determining CDx. The drug and the diagnostic, such as pharmacogenomic biomarker assays for patient selection, may reach the market at different times, delaying the implementation of precision medicine.

While recommendations on companion diagnostics are not issued by the EMA, the latest revision to the EU in vitro diagnostic medical devices legislation anticipates collaboration between medicines regulators and EU notified bodies, that conduct medical device conformity assessment, in conferring the CE label for novel companion diagnostics. In Q3, 2017, EMA released for public consultation a concept paper on the development and lifecycle of personalised medicines and companion diagnostics[11] - medical devices which allow prediction of the most likely response and adverse reactions that a particular patient may have when subjected to a specific treatment. The prospective guideline shall tender recommendations on the interface between predictive biomarker-based assays and medicinal products.

The European Medicines Agency (EMA) published guidance on the role of pharmacogenetic methodologies in the evaluation of drug pharmacokinetic properties and

[11] European Medicines Agency. Concept paper on predictive biomarker-based assay development in the context of drug development and lifecycle [Online]. EMA; 2017 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2017/07/WC500232420.pdf.

the Food and Drug Administration (FDA) published guidance on pharmacogenetics in early-phase clinical studies. Maliepaard and colleagues (2013) discuss the issues depicted by EU and US publications, along with related guidelines from the Pharmaceuticals and Medical Devices Agency (PMDA) in Japan, such as determining circumstances in which pharmacogenetic studies are recommended or required; the banking of trial participants' DNA allowing retrospective identification and testing with sufficient power; and the interpretation of knowledge in drug labelling, for instance, through dosing adjustment recommendations. Considerable differences exist in the approach adopted by the three agencies to deal with key issues in the application of pharmacogenetics to pharmacokinetic parameters (Maliepaard et al, 2013).

The EMA guidelines focus on pharmacokinetic parameters in preclinical and clinical phases I–IV. In Japan, the 2014 draft "Guidelines for Pharmacokinetic Drug Interaction for Drug Development and Proper Information Provision" followed the PMDA 2001 guidance on pharmacokinetic studies[12,13] covering clinical phases I–IV. The FDA emphasizes on early clinical phases (I–II), with recommendations for labelling included in the 2013 guidance for industry[14]. Divergences are noted among the three regions on when pharmacogenetic-related pharmacokinetic studies are recommended or required. EMA put forward decision-making guidance for early-phase drug development[15]: genotyping during first-in-human and further Phase I studies is required when in vitro data indicates that >50% of the drug is predicted to be cleared via a single polymorphic

[12] PMDA. Guideline on Clinical Pharmacokinetic Studies of Pharmaceuticals. Notification No. 796 [Online]. PMDA; Japan: 2001 [accessed 2019 Jul 28]. Available from: http://www.nihs.go.jp/phar/pdf/ClPkEng011122.pdf.
[13] PMDA. Guideline on Methods of Drug Interaction Study. Notification No. 813 [Online]. PMDA; Japan: 2001 [accessed 2019 Jul 28]. Available from: http://www.nihs.go.jp/phar/pdf/DiGlEngFinal011209.pdf.
[14] FDA. Guidance for Industry - Clinical Pharmacogenomics: Premarket Evaluation in Early-Phase Clinical Studies and Recommendations for Labeling [Online]. FDA; US: 2013 [accessed 2019 Jul 28]. Available from: https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM337169.pdf.
[15] European Medicines Agency. Guideline on the use of pharmacogenetic methodologies in the pharmacokinetic evaluation of medicinal products [Online]. EMA; 2011 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/02/WC500121954.pdf.

enzyme, and in dose-finding Phase II studies when a single functionally polymorphic enzyme is responsible for >25% of drug metabolism in vivo (Maliepaard et al, 2013). These in-vitro/in vivo cut-off values are not ratified by the respective United States or Japan agencies. In view of regional laws, regulations and ethics committees overseeing sample collection, data collection and data protection, the stringency of banking of DNA samples varies. The EMA highly recommends banking of DNA samples, while the FDA and PMDA encourage it. Harmonisation of such aspects should aid global drug development programmes by providing coherent, integrated and pragmatic guidance (Issa, 2002).

In recent years, Japan has seen revisions in the ethical guidelines for human genome and gene analysis, issues surrounding genetic testing have been recognized, and the PMDA has established a consultation system where clinical trial design and the applicability of pharmacogenomics markers are discussed during interview advice meetings (JPMA Regulatory Information Task Force, 2017). The FDA draft guidance issued in 2016[16] invited comments by stakeholders on the review of policies and procedures related to the genetic variant database and on recommendations to endorse publicly accessible human genetic variant databases as valid scientific evidence.

The Genetic Information Nondiscrimination Act characterises federal legislation concerning genetic technologies and data and took thirteen years from proposal date to being signed into law in 2008. Crafting legislation to respond to the successes of modern science and technology can indeed be a slow process. Many of the elements of the Genomics and Personalized Medicine Act introduced by Obama in 2006 and subsequent

[16] FDA. Use of Public Human Genetic Variant Databases to Support Clinical Validity for Next Generation Sequencing (NGS)-Based In Vitro Diagnostics - Draft Guidance for Stakeholders and Food and Drug Administration Staff [Online]. CDRH; US-FDA: 2016 [accessed 2019 Jul 28]. Available from: https://www.fda.gov/ucm/groups/fdagov-public/@fdagov-meddev-gen/documents/document/ucm509837.pdf.

versions of the bill which was not enacted into law were incorporated into, and expanded upon, in the 21st Century Cures Act[17] signed into law in 2016. The Act secures funding for the necessary scientific, medical, public health and regulatory infrastructure to expand sources of genetic information, support genomic-based technologies, streamline regulations and advance precision medicine.

1.1.10 The local scenario

Amitriptyline is a nationally authorised product (NAP), as is the case for the majority of medicines available in the EU, assessed through the national authorisation procedures of the respective Member States. Amitriptyline-containing medicinal products are authorised worldwide in more than 56 countries[18], including Malta where six authorisation holders registered a range of amitriptyline product names, with the national competent agency, the Medicines Authority[19]. Different brands may be accessible through local pharmacies at any point in time, subject to the companies' disposition to market the drug in Malta, considering the population size and corresponding demand.

Eurostat figures imply that chronic depression reports in Malta remained stable between 2002 and 2014. The Malta Health Interview Survey[20] recorded higher rates of depression in females than in males, 6.5% and 4.2% respectively, over the previous 12 months (2014-2015), with higher prevalence being reported for widowed or divorced persons and those

[17] 21st Century Cures Act, Pub. L. No. 114-255, 130 Stat. 1033 [Online]. US: 2016 [accessed 2019 Jul 28]. Available from: https://www.congress.gov/114/plaws/publ255/PLAW-114publ255.pdf.
[18] CHMP. Assessment report - Referral under Article 30 of Directive 2001/83/EC [Online]. EMA: 2017 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Saroten_30/WC500227970.pdf.
[19] Medicines Authority. Search for Malta Medicines [Online]. MA; Malta [accessed 2018 Jul 15]. Available from: http://www.medicinesauthority.gov.mt/search-medicine-results?modSearch=sim&field=A6CBAE9E8D6ADAD7F9802F8FF5.
[20] Directorate for Health Information and Research. Fact Sheet - 02/2017 [Online]. Ministry for Health; Malta: 2017 [accessed 2019 Jul 28]. Available from: https://deputyprimeminister.gov.mt/en/dhir/Documents/World%20Health%20Day%202017%20%20Depression%20let%27s%20talk.pdf.

of lower educational level. The 2015 European Health Interview Survey[21] highlights the substantial variability in the EU as for the percentage of individuals diagnosed with chronic depression. The highest percentage (12.1%) was reported in Ireland and the lowest in Romania (1.5%), with Malta ranking below the 7.1% EU average. Global health estimates by the World Health Organisation[22] indicate that 5.1% of the Maltese population suffer from depression, which translates to over 20,000 persons. Self-reported rates could represent an underestimation of actual figures, which may in turn reflect the country's approach to depressive disorders and their management.

The Malta Government Out-Patients Formulary List, updated by the Directorate for Pharmaceutical Affairs within the Ministry for Health[23], lists medicinal products that are available within the National Health Scheme for out-patients use, including antidepressants. Holders of a Pink Card (Schedule II), generally pertaining to limited means, are entitled for amitriptyline under criteria B (for both acute and chronic use). Amitriptyline is the only pink card positive antidepressant in the formulary classified for both short duration use and for an ongoing or recurrent condition. All medical practitioners working within the National Health Services and doctors practising privately may change the dose of treatment. The Fifth Schedule of the Social Security Act lists the diseases and conditions for which medicines are provided free of charge, irrespective of financial status. Once Schedule V entitlement is in place, amitriptyline may be issued via the Yellow Card. Tricyclic antidepressants (TCAs) are the only antidepressants in the formulary that may be initiated by any consultant rather than Consultant Oncologists,

[21] Eurostat. Mental health and related issues statistics [Online]. Updated September, 2017 [accessed 2018 Jun 29]. Available from: http://ec.europa.eu/eurostat/statistics-explained/index.php/Mental_health_and_related_issues_statistics.

[22] WHO. Depression and other common mental disorders: Global Health Estimates [Online]. Geneva: WHO; 2017 [accessed 2018 Jun 29]. Available from: http://apps.who.int/iris/bitstream/handle/10665/254610/WHO-MSD-MER-2017.2-eng.pdf;jsessionid=45C70DA60D1EDBDD6C543E54F44FB661?sequence=1.

[23] Directorate for Pharmaceutical Affairs. Out-Patients Formulary List [Online]. Ministry for Health; Malta: 2018 [accessed 2018 Jun 29]. Available from: https://deputyprimeminister.gov.mt/en/pharmaceutical/Documents/GFL/out_patients_gfl_may_2018.pdf.

Consultant Psychiatrists, and/or Consultant Palliative Care, as applicable to other antidepressants, particularly monoamine-oxidase inhibitors (moclobemide), selective serotonin re-uptake inhibitors (fluoxetine, fluvoxamine, paroxetine) and other antidepressant drugs (flupentixol and venlafaxine). Amitriptyline is analogously listed in Section 4.6.3 of the Government Out-Patients Formulary List, dedicated to neuropathic pain, which also includes nortriptyline, and pregabalin that is protocol-regulated for use in epilepsy, trigeminal neuralgia, and malignant diseases. Analgesics, both opioid and non-opioid, and non-steroidal anti-inflammatory drugs are also available for pain management through Malta's National Health System. In a 2017 survey on chronic pain[24], the average daily pain levels reported by Maltese respondents were the highest among 12 countries. Conversely, compared to countries like Spain, Norway and Germany, Maltese chronic pain patients report a shorter time period of pain between their first consultation with a healthcare professional and time of diagnosis, potentially supporting more timely initiation of therapy.

Patients entitled to amitriptyline through the public health sector are registered within the Pharmacy of Your Choice (POYC) Scheme and collect a regular two-month supply from their community pharmacy. Patients may be followed up by their respective clinicians, and a number of individuals have regular appointments at the Psychiatric Out-Patients (POP) or the Pain Clinic at Mater Dei Hospital, with examinations ranging from clinical assessment to routine bloods, cardiac investigations, and interventions as deemed necessary by the attending practitioners. Therapeutic drug monitoring is not standard local practice for patients on amitriptyline. Same applies for genotyping, which is mainly implemented in singular intricate cases, predominantly in oncology.

[24] Pain Alliance Europe. Survey on chronic pain 2017: Diagnosis, treatment and impact of pain [Online]. PAE-EU; Brussels: 2017 [accessed 2019 Jul 28]. Available from: http://www.pae-eu.eu/wp-content/uploads/2017/12/PAE-Survey-on-Chronic-Pain-June-2017.pdf.

1.2 Outline and rationale

Tricyclic antidepressants (TCAs) have been in clinical use for over fifty years and, owing to their efficacy and pharmacoeconomic advantage, are still prescribed for the treatment of major depression and other psychiatric conditions as well as in bulimia, enuresis and pain management (Dean, 2017). The interplay between genetic markers and environmental factors defining the heterogeneous depressive phenotypes and risk of developing depression, appears to be replicated in the ambit of antidepressant therapy outcomes. Efficacy and tolerability of TCAs is influenced by genetic polymorphisms of cytochrome P450 subfamily enzymes, particularly CYP2C19 and CYP2D6, that impact on metabolism and drug exposure, which in turn, may also be moderated by concomitant medication and individual pharmacokinetic variability. A limited number of reports have focused on methodological considerations for the analysis of amitriptyline and corresponding metabolites in blood, which could enable further understanding of the confounding factors influencing therapeutic drug monitoring results.

Clinical effectiveness is determined by how an antidepressant is used in everyday practice. Inadequacies in dosing regimens and applicable monitoring diminish the likelihood of favourable outcomes. This is particularly relevant for the tricyclics – naturalistic studies demonstrate that patients started on TCAs, as opposed to SSRIs, often receive sub-therapeutic doses for inadequate duration (Donoghue & Hylan, 2001). In neuropathic pain, caregivers may hesitate to titrate dosing as recommended and continue prescribing amitriptyline at the starting dose (Kamble et al, 2017), sensitized about the reported safety concerns, such as QT prolongation and cardiotoxicity.

Modern-day analytics, generating exploitable data on individual pharmacogenetics and pharmacokinetics may mitigate the uncertainty associated with the trial-and-error

prescribing of established drugs, such as amitriptyline, known to be among the most effective to date. Clinical application of gene-guided recommendations has started to be included in decision support structures, such as in the Netherlands, where pharmacogenetic guidance is integrated in the national drug database. Many other institutions, however, question whether the benefit of applying these recommendations alongside clinical judgement outweighs the inconveniences in the already complex clinical scenarios. Philosophical considerations, such as whether progress is being achieved towards sustainable innovation or else there is induction of interest in novel methodologies that healthcare systems cannot afford, may influence policy makers (Dove & Özdemir, 2013).

Scientific, financial, ethical and commercial hurdles, together with regulatory issues, represent an added concern in making precision medicine a working reality. The regulatory groundwork on the applicability of pharmacogenetics, as presented in guidance documents from the EMA, the FDA and the PMDA, complements scientific discussions between the regulatory bodies and innovators during drug development (Maliepaard et al, 2013). Drugs that are already registered may benefit from pharmacogenetic-related investigations triggered by observations in Phase IV of the drug's life cycle, and are possibly underserved by the regulatory framework. Along with supporting global harmonisation for genomic research in drug development, it may be rational to look into the applicability of the data we have today.

The putative status of infallibility that genetics fostered along the decades elicited exceptional influence on the pharmaceutical domain. Scientific advances and improved knowledge of the human genome have remodelled drug development while triggering changes in treatment paradigms and stakeholder expectations. Beyond the conceptual

confusion on whether medicine is becoming more precise, personalised, patient-centred, individualised, or stratified (De Grandis & Halgunset, 2016), the rationale, at least thus far, is not to treat each and every patient in a different way from every other patient. Genetic markers may be exploited for their potential to predict treatment outcomes, enabling the tailoring of doses for subjects who are likely to have a favourable response while identifying cases where risk of toxicity is conceivably high. The expanding body of knowledge necessitates dutiful interpretation to assess whether it can translate into tangible clinical outcomes.

A number of antidepressants may have 'informative PGx' within the respective labels, including reference to a gene or protein involved in the drug's metabolism or pharmacodynamics, but information to suggest the corresponding variation in the expected outcomes is limited. It is a general understanding that CYP genotyping is not markedly expedient for SSRIs, some of which are potent self-inhibitors of their metabolism. No clear-cut correlation between dosage, blood concentration and clinical outcomes has been demonstrated for SSRIs, which exhibit wide ranges between therapeutic and toxic doses, and elimination variation induced by genetics or the environment is presumed to be of limited clinical concern (Spina & de Leon, 2015).

On the other hand, tricyclic antidepressants, amitriptyline in particular, hold 'actionable PGx' in that, although genetic testing is not listed as a requirement in labels, altered blood levels and potential toxicity due to genetic variants in subsets of the population are recognised. The narrow therapeutic range and the relatively linear kinetics expected for amitriptyline were considered in selecting a research case example that would allow practical interpretation of serum concentrations in light of genotyping results. Acknowledging the recognised efficacy in depression, and the expanding data in

neuropathic pain, amitriptyline embodied the candidate drug for this study, to assess whether advancements in the pertinent regulatory, analytical and clinical fields, may support better informed use of this established drug.

The prospect of further research on tricyclics is unpromising, with drug companies showing tapered interest, particularly in depression. Industry is reassured by the widespread use of available antidepressants and perceives no financial or regulatory pressure for further data. Maximising drug potential in real-world scenarios depends on the adoption of applicable pharmacogenetic evidence and analytical findings in a multi-disciplinary clinical setting, supported by progressive regulatory initiatives for integration in official product literature and throughout the evaluation of safety concerns.

Education in pharmacotherapy, coupled with knowledge in pharmacokinetics, pharmacogenomics and related informatics, should empower pharmacists to play a leading role in the clinical implementation of pharmacogenetics. The intricate implications of pharmacogenetics in amitriptyline therapy embody the challenge this project embarked to address. The research was designed to incorporate regulatory, analytical and clinical aspects in determining the pragmatic implications of pharmacogenetics for delivering an established drug through individualised therapy with minimal risk.

1.3 Research questions, aims and objectives

Research question I ***Regulatory***

Is the regulatory infrastructure supporting the implementation of precision pharmacotherapy by integrating pharmacogenetics in the product information and throughout the evaluation of safety concerns?

Aim To appraise harmonisation in the integration of pharmacogenetic implications in official sources of amitriptyline information, and pharmacovigilance activities undertaken for amitriptyline.

Objectives

i. To assess the inclusion of pharmacogenetic information in the amitriptyline Summary of Product Characteristics.

ii. To evaluate pharmacogenetic considerations in the reporting and assessment of suspected amitriptyline drug interactions.

Research question II ***Analytical***

What are the analytical and technical requisites for the application of a pharmacogenetic approach to guide decisions in practice?

Aim To study the investigative means (chemical/genetic) and interpretational measures that facilitate construal of potential associations between blood levels, genotype and confounding factors in the course of treatment with amitriptyline.

Objectives

i. To develop a laboratory method for measuring levels of amitriptyline, nortriptyline, and their hydroxy-metabolites in serum.

ii. To perform *CYP2C19* and *CYP2D6* genotyping and interpretation of inferred phenotypes.

Research question III ***Clinical***

Does the assessment of dose-related reference ranges, metaboliser status and phenoconversion potential facilitate the interpretation of therapeutic drug monitoring and clinical outcomes alongside genotype-guided dosing recommendations?

Aim To investigate the relationship between genetic variability in CYP450 enzymes, metaboliser status and adverse events during amitriptyline therapy, through genotypic, serum concentration and side-effect data.

Objectives

i. To examine the influence of CYP2C19 and CYP2D6 genotype and concomitant CYP inhibitors, on serum concentrations of metabolites/parent-drug, evaluated in tandem with dose-related reference ranges.

ii. To explore potential links between metaboliser status, therapeutic drug monitoring outcomes and side-effects reported by patients on amitriptyline.

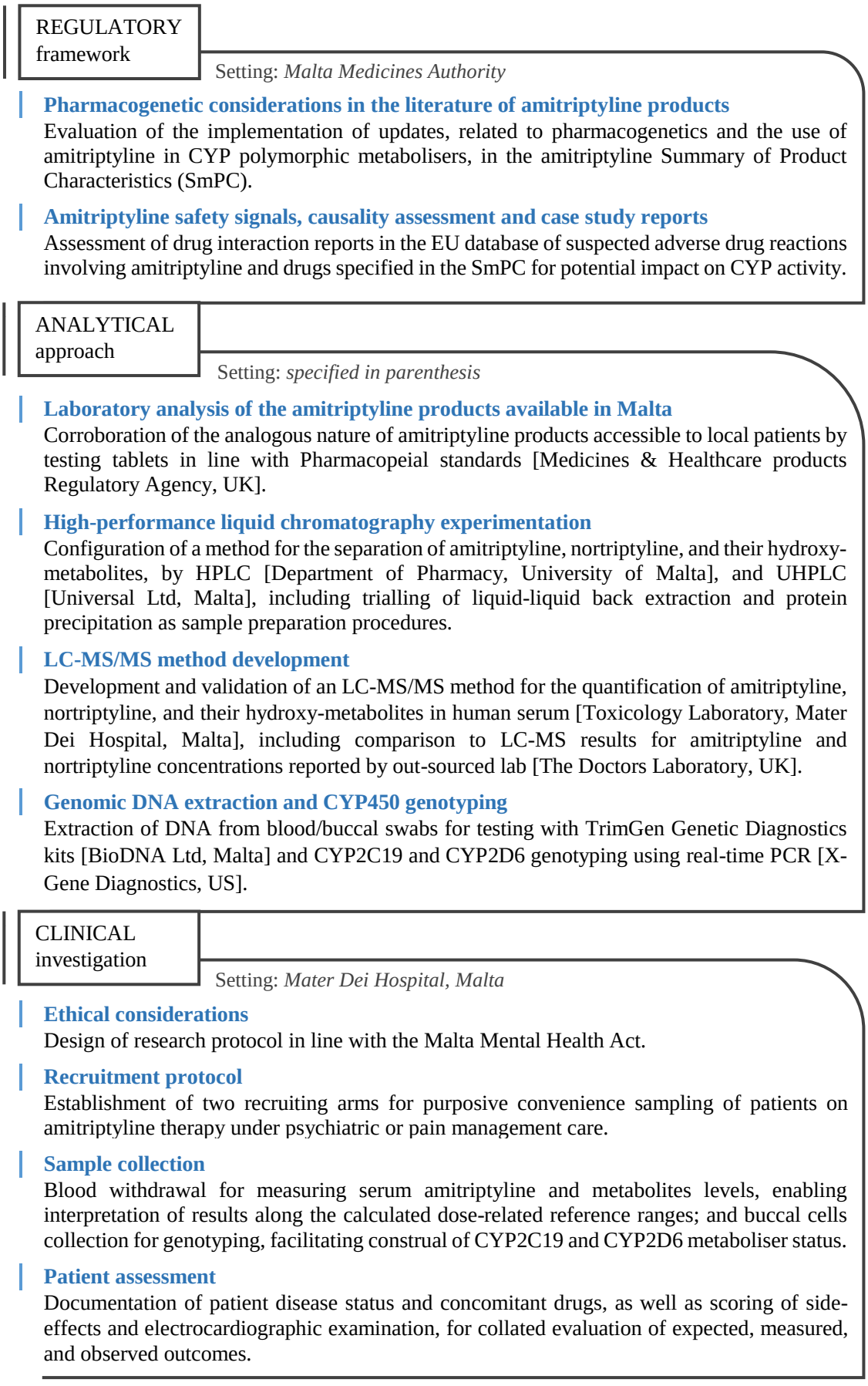
Chapter 2
Methodology

2.1 Research design

It was reflected in Chapter 1 that the use of established medicinal products, such as tricyclic antidepressants (TCAs), derives from a time when genotyping studies were not available. Pharmacogenetics embodies an interesting lead to investigate whether drug potential could be maximized through reattribution of medical use for drugs in which the balance between efficacy and toxicity is difficult to strike in the general population. Practical analytical technologies, conversant regulatory developments and clinical implementation experience, or the lack thereof, are primary clinchers, in the enduring endeavours to realise the translational quality of pharmacogenetics. Inferences from genotyping and pharmacokinetic considerations may support the careful exploitation of the benefits of amitriptyline and serve to update product information. The relevance of therapeutic drug monitoring and informed assessment of adverse drug reactions may be revisited, with the generation of interpretable data and enhanced prescriber awareness.

TCAs constitute relevant candidates for studying the implications of genotype-guided prescribing, considering that the effect of *CYP2D6* and *CYP2C19* polymorphisms on individual amitriptyline and metabolites exposure may predispose to adverse events or treatment failure. The ratio between parent drug and relevant metabolites may allow better understanding of potential correlations between genotype, metaboliser status and clinical outcomes. This work was designed to investigate the case of amitriptyline, considering the implications of pharmacogenetics from a multidisciplinary viewpoint – regulatory, analytical, and clinical. A range of settings and resources, both local and international, is entailed for the integrated approach adopted to address the research questions. The methodology is subdivided according to the aspect under study (Figure 2-1), followed by details on the way data processing was carried out.

Figure 2-1: Synopsis of methodology



During the course of this research, parts of the material presented herein have been disseminated to support timely sharing of information across the scientific community. A list of publications and abstracts is included at the end of this work.

2.2 Regulatory framework

In light of the evolving regulatory context and the impact it has on the clinical application of pharmacogenetics, this area of study was taken up to understand the state of affairs and to develop an informed approach in addressing the subject matter. A concise overview of specific regulatory procedures is included (Section 2.2.1), to enable explanation of how the regulatory framework was studied to evaluate pharmacogenetic considerations in the Summary of Product Characteristics (SmPC) and across reports concerning safety implications of amitriptyline, as the case example of interest. The two-fold appraisal consisted of assessing the level of harmonisation reached on the integration of pharmacogenetic implications in the official sources of amitriptyline information (Section 2.2.2), and evaluating whether aberrant metabolism, and possible causative interactions, are given consideration in pharmacovigilance activities undertaken for amitriptyline (Section 2.2.3).

2.2.1 Contextual background and data sources

As primer to Section 2.2.2 – Pharmacogenetic considerations in the literature of amitriptyline products – it is worth appreciating that the product information for amitriptyline was given notable attention over the past years, particularly since 2015. In the EU, Saroten (and associated names such as Redomex) is marketed as the originator

product for amitriptyline, by Lundbeck A/S group and associated companies[25]. As part of the amitriptyline Periodic Safety Update Report (PSUR) Single Assessment (PSUSA) procedure (PSUSA/0000168/201501), Greece, as the Lead Member State being assigned amitriptyline, identified the need to harmonise the Summary of Products Characteristics (SmPCs) for Saroten and associated names (amitriptyline) across the EU. Divergences between the SmPCs approved in the EU Member States for Saroten and other amitriptyline containing products were recognised with respect to indications, posology, contra-indications, undesirable effects, and other sections. The Greek National Competent Authority notified the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency on 17 December 2015 of a referral under Article 30 of Directive 2001/83/EC[26] for Saroten and associated names.

Insight on the procedures involved in the invocation of Article 30 is paramount to understanding the methodology implied in evaluating the evolvement of amitriptyline product information, as reflected through EU regulatory sciences. In Article 30 "harmonisation" referral procedures[27], Marketing Authorisation Holders (MAHs) are informed of the procedure initiation, together with the notification triggering the procedure, the timetable and the list of questions adopted by CHMP. MAHs are requested to submit relevant information for assessment by the CHMP, including proposals for a harmonised summary of product characteristics (SmPC), labelling and package leaflet (PL). Additional data may be collected by the CHMP through a list of outstanding issues and in an oral explanation. The assessment of all the available data results in the CHMP

[25] European Medicines Agency. Fourth CHMP list of outstanding issues - To be addressed by the marketing authorisation holders for Saroten and associated names; Procedure no: EMEA/H/A-30/1430. EMA/CHMP/853474/2016.

[26] Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use. *Official Journal* 2001;L311:67.

[27] European Medicines Agency. Questions & answers on Article 30 referral procedures [Online]. EMA; 2016 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Regulatory_and_procedural_guideline/2016/07/WC500209672.pdf.

adopting an opinion on the issue reviewed. The European Commission then starts the decision-making process leading to the adoption of a binding decision addressed to the Member States and notified to the MAHs. Member States concerned by the referral have an obligation to implement the harmonised SmPC, PL and labelling of the Commission decision, as applicable. It is recommended that MAHs of the medicinal products covered by the defined scope of the Commission Decision submit a variation within 10 days after Commission Decision, which is announced in the Co-ordination group for Mutual recognition and Decentralised procedures – human (CMDh) press release to remind MAHs of generic medicinal products to implement the outcome of the referral procedure through the submission of the relevant variation[28].

For this research, data sources on the amitriptyline referral procedure and variation implementation were accessible through the European and national regulators respectively. Japan and US regulators were also contacted to add a brief international perspective, which may be of interest to probe the global harmonisation work-in-progress, at a time when European regulatory sciences are not fully harmonised, as outlined in sections 2.2.2 of the methodology and 3.1.1 of the results.

Comprehension of post-marketing safety surveillance in the EU serves as background to Section 2.2.3 – Amitriptyline safety signals, causality assessment and case study reports. Marketing Authorisation Holders are required to submit, to the regulatory authorities, a report on the benefit-risk evaluation of authorised medicines. MAHs submit electronic Periodic Safety Update Reports (PSURs) to the EMA, which may be accessed, through a

[28] CMDh. Recommendation for implementation of Commission Decisions or CMDh agreements following Union referral procedures where the Marketing Authorisation is maintained or varied [Online]. EMA; 2014 [accessed 2019 Jul 28]. Available from: http://www.hma.eu/fileadmin/dateien/Human_Medicines/CMD_h_/procedural_guidance/PostReferral_Phase/CMDh_318_2014_Rev.00_2014_09.pdf.

repository, by Member States. PSUSAs - single assessments of EU PSURs - are carried out per drug substance. All Marketing Authorisation Holders for products comprising of a specific substance, submit PSURs simultaneously to be assessed by the Pharmacovigilance Risk Assessment Committee (PRAC). The PSURs assessed by the PRAC cover centrally and nationally authorised products, including through the mutual recognition and decentralized procedures (Borg et al, 2015), as is the case for amitriptyline. The PSUR assessment under a PSUSA procedure for nationally authorised products (NAPs) is summarised in Figure 2-2.

Figure 2-3 gives an overview of the exchange of information for NAPs in the EU signal management process. The EMA defines safety signals as 'information on a new or incompletely documented adverse event which is potentially caused by a medicine and warrants further investigation' (European Medicines Agency, 2017). Signal detection is legally mandated and carried out for all products by the EU network, as well as the MA holders, with national competent authorities (NCAs) leading for nationally authorised products (NAPs) through work sharing. Greece is the EU Member State responsible for amitriptyline. PRAC recommendations for NAPs necessitating regulatory action, such as the amendment of product information are submitted to CMDh, the Co-ordination Group for Mutual Recognition and Decentralised Procedures – Human, for information. Variations, submitted by MAHs for NAPs are processed at national level and Member States are responsible to oversee that PRAC recommendations on signals[29] are implemented.

[29] Pharmacovigilance Risk Assessment Committee. PRAC recommendations on signals [Online]. EMA; 2018 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/PRAC_recommendation_on_signal/2018/04/WC500247424.pdf.

Figure 2-2: Periodic Safety Update Report (PSUR) Single Assessment (PSUSA) procedure for nationally authorised products [30]

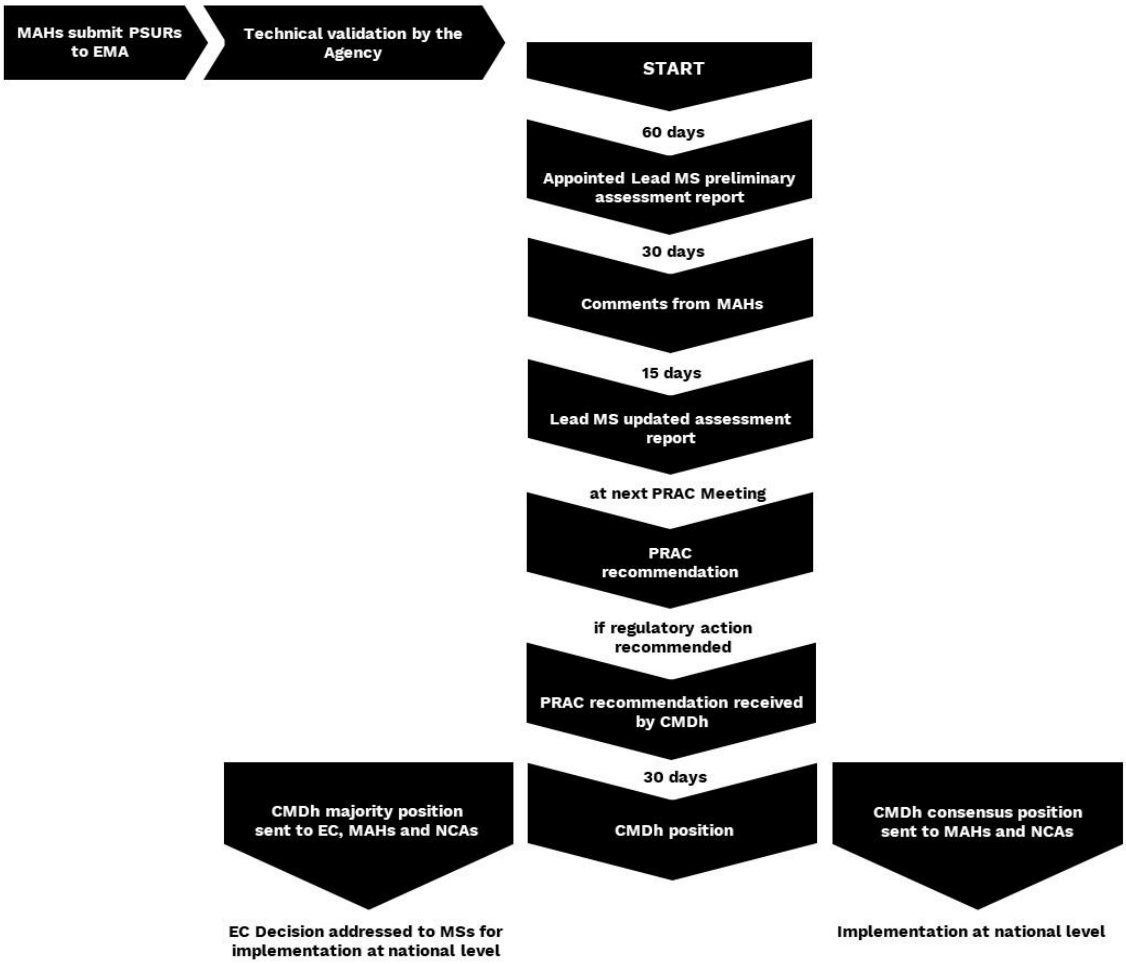
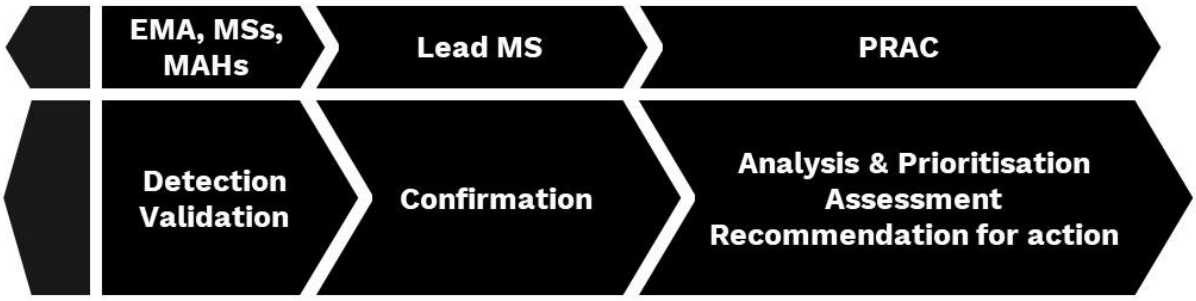


Figure 2-3: EU signal management process for nationally authorised products



[30] European Medicines Agency. Periodic Safety Update Reports: questions and answers [Online]. EMA [accessed 2018 Aug 18]. Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/q_and_a/q_and_a_detail_000041.jsp&mid=WC0b01ac0580023e7d.

Screening of adverse reactions in Eudravigilance[31], EMA's database of suspected ADRs, exploits tools comprising of the electronic Reaction Monitoring Report (eRMR) which provides summarised signal detection data and the EudraVigilance Data Analysis System (EVDAS) for detection and signal management activities. The Proportional Reporting Ratio (PRR) analysing the proportion of all reactions to a drug in comparison to the same proportion of all drugs in the database, has been implemented in the signal detection method in EudraVigilance. The PRR is used to detect signals of disproportionate reporting (SDRs) in pharmacovigilance. Disproportionality statistic and other data, such as the number of reports submitted, are adopted to indicate when further inspection is warranted for a given drug-event combination.

Online resources, as well as the EudraVigilance database, were central sources of data in this research, as outlined in sections 2.2.3 of the methodology and 3.1.2 of the results. The Malta Medicines Authority facilitated access to documents, as well as active participation on the scientific committees and working parties of the European Medicines Agency. Contact was established with colleagues from the Greek competent authority, including Dr Agni Kapou, PRAC member for Greece, and developments were followed throughout the course of study. Presentations delivered at PRAC meetings throughout the research period were reviewed for content related to amitriptyline, and procedures at EU level shadowed through follow-up with the Greek rapporteur for amitriptyline, and the PRAC member and alternate of the Malta Medicines Authority, with insights from Professor John Joseph Borg. Regulatory procedures related to amitriptyline were followed as they progressed through EMA's Pharmacovigilance Risk Assessment Committee (PRAC), Committee for Medicinal Products for Human Use (CHMP),

[31] European Medicines Agency. Screening for adverse reactions in EudraVigilance [Online]. Inspections, Human Medicines, Pharmacovigilance and Committees Division; EMA:2016 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Other/2016/12/WC500218606.pdf.

Coordination Group for Mutual Recognition and Decentralised Procedure - human (CMDh) and the European Commission. In understanding the pharmacovigilance activities on an international level, data was requested from the FDA (US) and PMDA (Japan) on adverse drug reaction reports received in relation to amitriptyline.

2.2.2 Pharmacogenetic considerations in the literature of amitriptyline products

The entire Article 30 referral procedure for amitriptyline was followed, with particular interest for updates related to pharmacogenetics and the use of amitriptyline in CYP polymorphic metabolisers. The implementation of the outcomes of the referral was reviewed by evaluating corresponding revisions in the Summary of Product Characteristics (SmPCs) of amitriptyline products. According to the list of national authorisations[32] published for PSUSA/00000168/201501, UK has a significant number of authorised amitriptyline products. The term 'amitriptyline' was searched in the medicines database of the United Kingdom (UK) Medicines and Healthcare Products Regulatory Agency (MHRA) website [http://www.mhra.gov.uk/spc-pil/] in August 2018, 15 months following the European Commission implementing decision of May 2017. The search yielded 33 different Marketing Authorisation Numbers with their corresponding SmPCs, which were assessed by direct inspection. In view of the ubiquitous preoccupation with respect to global harmonisation, this exercise was undertaken to review, at the outset, the level of accordance within EU Member States.

For an international perspective, the Division of Drug Information in the FDA's Center for Drug Evaluation and Research was contacted to gain further insight on the source of

[32] European Medicines Agency. List of nationally authorised medicinal products - Active substance: amitriptyline Procedure no.: PSUSA/00000168/201501 [Online]. EMA; 2015 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Periodic_safety_update_single_assessment/2016/10/WC500214456.pdf.

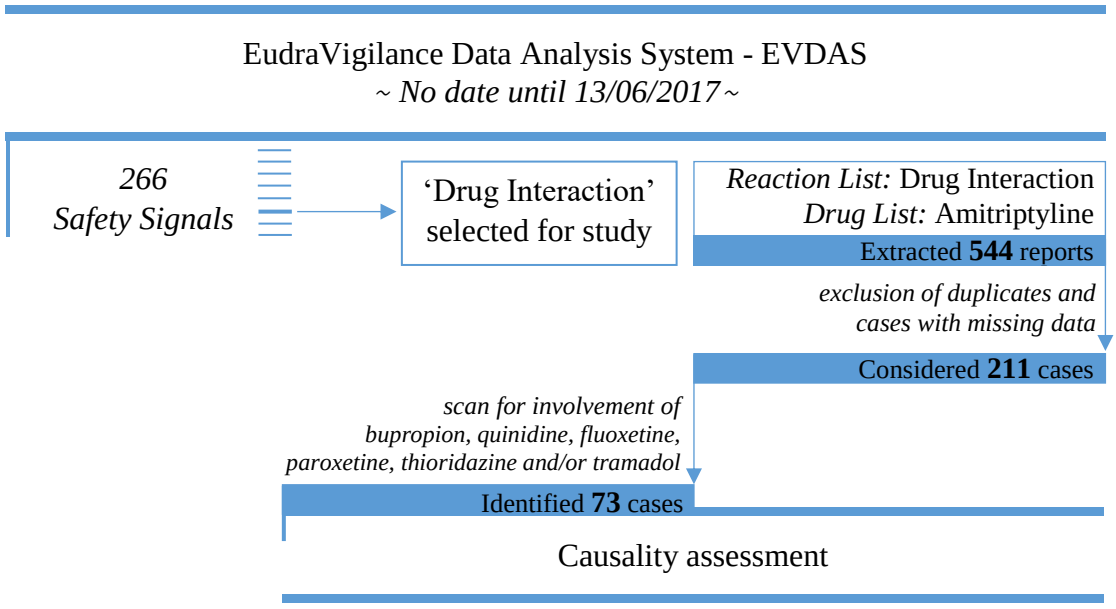
information on amitriptyline in the US. The FDA Division of Drug Information identified drug labeling, also called the prescribing information (PI), as the single source of information on any medication in the US. Contact was established, with the Pharmaceuticals and Medical Devices Agency (PMDA), Japan, to assess amitriptyline package inserts, which, for clinical drugs regulated by the Japanese Pharmaceutical Affairs Law, represent the legal source of information, whereas supplementary information is provided in interview forms (Ohno et al, 2006). The PMDA provided a link [http://www.pmda.go.jp/english/search_index.html] to the package inserts and interview forms of drugs approved in Japan. PMDA recommended that the two Marketing Authorisation Holders of amitriptyline products in Japan are contacted directly. Sawai Pharmaceutical (MAH for Amitriptyline Hydrochloride tablet) and Nichi-Iko Pharmaceutical (MAH for Tryptanol tablets) were emailed twice in Aug/Sept 2017 but no response was received.

2.2.3 Amitriptyline safety signals, causality assessment and case study reports

The static Proportional Reporting Ratio (PRR) evaluation for amitriptyline extracted through the EudraVigilance Data Analysis System (EVDAS) with an inclusive date range spanning to June 2017 (Figure 2-4), identified 266 safety signals for amitriptyline, with reported adverse effects ranging from somnolence to dry mouth, tachycardia, and coma. 'Drug Interaction' was selected as the reaction under study. The five-hundred fifty-four (554) reports that included 'Drug Interaction' in the *Reaction List* and 'amitriptyline' in the *Drug List* were retrieved. Duplicates and cases with missing data (e.g. narrative or list of concomitant drugs absent) were excluded and a total of two-hundred twenty-one (221) cases were considered.

The amitriptyline Summary of Product Characteristics (SmPC) infers that monitoring TCA plasma levels should be considered whenever amitriptyline 'is to be co-administered with another drug known to be an inhibitor of CYP2D6' since 'dose adjustment may be necessary'. The SmPC for amitriptyline specifically identifies bupropion, quinidine, fluoxetine and paroxetine as strong CYP2D6 inhibitors. It states that 'these drugs may produce substantial decreases in TCA metabolism and marked increases in plasma concentrations'. Reference is made to the CYP2D6 substrates thioridazine and tramadol which, if co-administered with amitriptyline, may have their metabolism inhibited, resulting in increased risk of side-effects. A search for these drugs was made in the 221 cases considered and 73 cases were identified which involved bupropion (6), quinidine (1), fluoxetine (13), paroxetine (17), thioridazine (2), and/or tramadol (42). Other drugs, which may also impact CYP activity and corresponding pharmacokinetics, but are not specifically mentioned in the SmPC, were not taken in consideration. The CIOMS forms (ADR reports in the format established by the Council of International Organisation of Medical Sciences) related to these cases were extracted to support review.

Figure 2-4: Data extraction for assessment of drug interaction cases



The reports were evaluated to assess whether drug interaction cases submitted for amitriptyline with concomitant administration of one of the CYP2D6 substrate/inhibitor may be linked to a potential interaction caused by altered enzymatic metabolism or other causes, such as inappropriate dosing or genetic variation in *CYP2D6*, may be more plausible. The causality was judged on the data present in the case and the drug-reaction pairs presented by the patient. The assessment procedure was constructed on the basics of established methods, particularly the French Method for causality assessment of ADRs (Hire et al, 2013), adapted for the purpose of the exercise. The criteria included: (i) chronology time-sequence analysis considering challenge, dechallenge and rechallenge; (ii) semiology signs and symptoms considering pharmacological plausibility, other causes for event and laboratory test results.

Cases were scored as follows:

- Highly probable: a clinical event, which cannot be explained by concurrent disease, occurring in a plausible time relative to the administration of amitriptyline and the CYP2D6 substrate/inhibitor, with dechallenge (the response to withdrawal of the drug/s) being clinically plausible, and with a satisfactory rechallenge procedure; or

- Probable: a clinical event, unlikely to be attributed to concurrent disease, with a reasonable time sequence to the administration of amitriptyline and the CYP2D6 substrate/inhibitor, which follows a clinically reasonable response on dechallenge; or

- Possible: a clinical event, with a reasonable time sequence to the administration of amitriptyline and the CYP2D6 substrate/inhibitor, but which could also be explained by concurrent disease; information on drug(s) withdrawal lacking or unclear; or

- Unlikely/Uncertain: clinical event with a temporal relationship to the administration of amitriptyline and the CYP2D6 substrate/inhibitor which makes a causal relationship improbable, and in which underlying disease provides possible explanations.

Other conceivable causes, such as individual genetic variation resulting in altered metabolism, were considered, in the line with goal of determining whether a pharmacokinetic interaction at the metabolising enzyme level, and a potential corresponding alteration in blood concentrations, was the most plausible cause or otherwise.

As an auxiliary exercise enthused by the emergent observations from the clinical investigation in this research, Individual Case Study Reports (ICSRs) for amitriptyline and "dry mouth" or "sedation" as Preferred Terms (PTs, distinct descriptors for symptom/sign), were extracted, from the EU database on ADRs - EudraVigilance, using the EudraVigilance Data Analysis System (EVDAS). Adverse drug reaction (ADR) data retrieved was rationalized to determine the number of reports for dry mouth and for sedation, according to the daily dose administered. Reports which did not specify the dosage of amitriptyline were excluded from the dataset.

On an international exploratory level, the PMDA informed that reports of adverse reactions submitted in the post-marketing phase are retrievable from their dedicated webpage[33]. In reply to the same enquiry submitted to the FDA, the Center for Drug Evaluation and Research identified the section 'Post-marketing Adverse Events' in the product labelling as a good source for pharmacovigilance information on any drug marketed in the United States. The FDA Adverse Event Reporting System (FAERS) database[34], containing information on adverse event and medication error reports submitted to the FDA, was referred to in the response provided. It was however indicated that the database, designed to support the FDA's post-marketing safety surveillance

[33] Pharmaceuticals and Medical Devices Agency. PMDA [Online]. Japan [accessed 2019 May 26]. Available from: http://www.pmda.go.jp/safety/info-services/drugs/adr-info/suspected-adr/0005.html.
[34] Food and Drug Administration. FDA [Online]. US [accessed 2019 May 26]. Available from: https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Surveillance/AdverseDrugEffects/ucm082193.htm.

programme for drug and therapeutic biological products, contains raw data that does not support a search through the files since users need to be familiar with creation of relational databases.

2.3 Analytical approach

Physicochemical data on amitriptyline and corresponding metabolites was retrieved to inform ensuing method development for laboratory determination of concentrations in human serum. Amitriptyline is made up of a hydrophobic skeleton and a basic tertiary amino group as the main functional group. The compound, 3-(10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-ylidene)-propyldimethylamine, with a molecular weight of 277.39, is practically insoluble in water as the free base. Amitriptyline hydrochloride is freely soluble in alcohol and water and easily dissolved in polar solvents such as acetonitrile. Water solubility of the salt is pH-dependent with the highest solubility in an acidic environment. Nortriptyline, 3-(10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5-ylidene)-N-methyl-1-propanamine, is a secondary amine with a molecular weight of 263.37. Tricyclics possess similar structures, mass and pKa values, implying similar chromatographic behaviour, and separation may be difficult (Manzo et al, 2006). Besides the chemical aspect of the analytical approach, intricacies were also foreseeable in the genetic aspect to avert inconsistent interpretations in view of the large number of polymorphisms to be assayed and the occurrence of copy number variations.

2.3.1 Purpose and resources

Methodical considerations were studied for determining serum amitriptyline concentrations and ratios of metabolites to parent drug and for testing *CYP2C19* and

CYP2D6 genetic polymorphisms. The approach adopted was intended to develop the means that would subsequently facilitate understanding of possible correlations between blood levels, genotype and other potentially confounding factors in patients on amitriptyline therapy. A collaborative framework centred on the pooling of resources was established, as summarised in Figure 2-1.

2.3.2 Laboratory analysis of the amitriptyline products available in Malta

Amitriptyline is a highly lipophilic molecule with the hydrochloride being described as freely soluble in water[35]. Amitriptyline is well absorbed but extensive first-pass metabolism moderates the oral bioavailability to barely 50% (Schulz, 1985). Permeability is high and commonly used excipients are unlikely to have any effect on the bioequivalence of amitriptyline hydrochloride products. Manzo et al, 2006, recommend a waiver of in vivo bioequivalence testing for the approval of amitriptyline hydrochloride immediate release solid oral dosage forms provided that the formulation excipients are in use in approved products and in vitro dissolution meets the criteria defined in Biopharmaceutics Classification System guidance (Manzo et al, 2006).

In Malta, the hydrochloride salt of amitriptyline is in therapeutic use as solid dosage forms with strengths of 10 mg or 25 mg. Since patients may access different brands of amitriptyline, the products available locally were identified through contact with the local agents and the Central Procurement and Supplies Unit within the Ministry for Health. A sample of each of the amitriptyline hydrochloride tablets, in the doses marketed under the respective trade names, was submitted for analysis in October 2017, through the Malta Medicines Authority, and tested at the MHRA Laboratory at LGC in Middlesex, UK.

[35] Ph. Eur. 9th edn. 2017. Amitriptyline hydrochloride, monograph 0464. Strasbourg, France, Council of Europe.

The products were analysed, in line with British Pharmacopeia 2017, for: (i) uniformity of weight - individual weights of twenty tablets should not deviate from the mean by ±10% (< 80mg), ±7.5% (80mg < weight < 250mg), ±5% (> 250mg); (ii) assay by HPLC - 90.0% to 110.0% of the stated amount as pass/fail criteria; and (iii) related substances by thin-layer chromatography - pass/fail criteria based on intensity of any secondary spots. Dissolution was tested, as per United States Pharmacopoeia 2017 - level S1 (six tablets), level S2 (12 tablets), with quantification by UV. A sample, incidentally one from a product batch with the closest expiry date (within 8 months of testing), failed dissolution on level S1 but passed on S2. All samples complied with the limits.

2.3.3 High-performance liquid chromatography experimentation

Data on the physicochemical properties of amitriptyline and corresponding metabolites was retrieved through online resources, particularly the Reaxys® (Elsevier) database. Amitriptyline has a pKa value of 9.4 and is unionized at high pH values. In reversed-phase high performance liquid chromatography, amitriptyline has a strong retention. Ionic interactions with the silanol groups of the stationary phase arise through the tertiary amino group. Since a basic environment is not suitable for most silica based column packing materials, the working pH typically results in amitriptyline being chromatographed in cation form. A relatively high content of organic modifier is expected for the mobile phase due to the highly hydrophobic nature. Amitriptyline absorbs UV light with the chromophore of the benzene ring being extended with the double bond in the side chain (Hansen et al, 2012). At a pH below the values of their pKa, the tricyclics are positively charged and interactions with silanol groups of the stationary phase may result in peak broadening and lack of selectivity and efficiency (Dell'Aquila, 2002).

HPLC method development was carried out at the Department of Pharmacy, University of Malta, starting with work on the chromatographic separation of amitriptyline and nortriptyline. Amitriptyline hydrochloride and nortriptyline hydrochloride standards were procured from the European Directorate for the Quality of Medicines (EDQM). Clomipramine hydrochloride standard was sourced from Sigma-Aldrich, Saint Louis, USA. Fisher Chemical, Leicestershire, UK, supplied HPLC-grade water and orthophosphoric acid. Acetonitrile was acquired from Carlo Erba Reagents, Val-de-Reuil, France, and disodium hydrogen phosphate from Scharlau, Sentmenat, Spain.

Solutions of 100 µg/mL amitriptyline and nortriptyline were prepared in HPLC-grade water and mixed together by transferring 0.5 mL of each solution into amber-coloured vials which were stored at 4 ˚C until analysed. Mobile phases consisted of acetonitrile and phosphate buffer, which were degassed in an ultrasonic bath before use. The buffer solution was prepared by dissolving 5.23 g extra pure, anhydrous, disodium hydrogen phosphate in 1 L HPLC-grade water. The pH (4.4, 5.6 or 6.8) was adjusted by dropwise addition of HPLC-grade orthophosphoric acid. Standard Hanna® calibrator buffer solutions were utilised, at pH 4.01 and 7.01, to calibrate the Hanna® Bench-top pH meter HI8521 used for pH measurements. Percentage acetonitrile composition was set at 30, 35 or 40%. An Agilent 1260 Infinity Series® II liquid chromatography system was employed for analysis. Amitriptyline and nortriptyline sample mixtures of 20 µL were injected. Triplicate runs were performed for each mobile phase composition using a Kinetex® C18 LC Column (150 x 4.6 mm; particle size 5 µm) at a temperature of 27 ˚C. The mobile phase flow rate was set at 1 mL/min. The UV wavelength was set at 210 nm. Separate runs for pure amitriptyline hydrochloride and nortriptyline hydrochloride were first performed to assist with peak identification.

Calibration in HPLC methods entails the use of an internal standard which is chemically similar to the targeted substances and can be completely separated from the peaks of other sample components. The mobile phase composed of acetonitrile 65:35 v/v, at pH 5.6, was employed to explore the implications of adding clomipramine, 3-(2-chloro-5,6-dihydrobenzo[b][1]benzazepin-11-yl)-N,N-dimethylpropan-1-amine, with a pKa of 9.2, for internal standardisation in the practical application of the method presented. A 0.5 mL aliquot of 100 µg/mL clomipramine hydrochloride solution, prepared by dissolving standard clomipramine hydrochloride powder in HPLC-grade water, was added to the amitriptyline and nortriptyline sample mixture. A 20 µL volume of the resulting mixture was injected into the same chromatographic system. A UV wavelength of 240 nm was also tested in this scenario. Serial dilutions of the amitriptyline and nortriptyline standard solutions were performed to obtain the following six concentrations: 5 ng/mL, 50 ng/mL, 100 ng/mL, 300 ng/mL, 500 ng/mL and 1000 ng/mL. Each of these solutions was analysed, through three consecutive runs in the system, to plot a calibration graph of average area under the peak against concentration.

The method developed for the separation of amitriptyline, nortriptyline and clomipramine, comprehensively described in the published article[36], was progressed further by studying the chromatographic conditions that would allow simultaneous determination of the hydroxy-metabolites: E-10-hydroxyamitriptyline, Z-10-hydroxyamitriptyline, E-10-hydroxynortriptyline and Z-10-hydroxynortriptyline. Clearsynth Labs Ltd, Mumbai, India, synthesized cis-10-hydroxyamitriptyline, and provided cis-10-hydroxynortriptyline and trans-10-hydroxynortriptyline. Trans-10-hydroxyamitriptyline was sourced from Sigma-Aldrich, Saint Louis, USA. A similar

[36] Mifsud Buhagiar L, et al. Implications of mobile phase composition and pH on the chromatographic separation of amitriptyline and its metabolite nortriptyline. *Int J Pharm Pharm Sci* 2018;10(4):132-8. Available from: https://innovareacademics.in/journals/index.php/ijpps/article/view/24817.

procedure was adopted, whereby reversed phase-HPLC parameters, particularly flow-rate and mobile phase composition and pH were fine-tuned to obtain optimal separation of the six tricyclic compounds and the internal standard.

Through collaboration with Universal Limited at the Malta Life Sciences Park, the developed method was adapted to an Ultra-High Performance Liquid Chromatography (UHPLC) system, to investigate whether the higher separation efficiency and sensitivity of UHPLC would improve peak resolution and potential quantification of the lower concentrations. Agilent 1290 Infinity LC with a Kinetex® 1.7 μm EVO column was used to run a set of the standard dilutions. The injection volume was 10 μL with a flow rate of 0.25 mL/min at a temperature of 27°C. The mobile phase was unchanged, consisting of phosphate buffer (pH 5.6) and acetonitrile.

In view of the prospective application of the developed method for the quantitative measurement of amitriptyline and metabolites in serum, investigation of sample preparation procedures ensued. Routine assays for bioanalysis have a sample preparation step involving protein precipitation, liquid–liquid extraction or solid phase extraction, which enable removal of proteins from the sample (Pandey et al, 2010). The removal of interferences from the sample matrix is expected to improve the compounds' stability and subsequent analytical performance. Sample preparation procedures often involve manually intensive protocols considered to be the part that is most time-consuming and prone to error (Ramos, 2012, Moein et al, 2017).

Dilutions of standard solutions of amitriptyline, notrtiptyline and hydroxy-metabolites at concentrations of 5 ng/mL, 50 ng/mL, 100 ng/mL, 300 ng/mL, 500 ng/mL and 1000 ng/mL were prepared. Human pooled serum, obtained from the Pathology Department

of Mater Dei Hospital, was spiked with the standard solutions and experimentation with liquid-liquid back extraction and protein precipitation followed.

Liquid-liquid back extraction consisted of three phases:

Phase 1

Clomipramine (50 µL of 100 µg/mL internal standard solution) was added to 1mL of serum spiked with 50 µL standard solution, mixed with 1 mL of 0.5M NaOH; 8 mL of butyl chloride were added to the tube, placed in rotator for 10 minutes of gentle shaking, followed by centrifugation at 3000 rpm for 5 minutes in Z446K (HERMLE Labortechnik GmbH, Germany).

- At pH 13.7 (upon addition of NaOH), amitriptyline and nortriptyline hydrochlorides are converted into the free amines which are soluble in the organic layer and extracted into the butyl ether, while water contaminants remain in the aqueous solution.

Phase 2

The organic layer was transferred to another tube, 2.5 mL of 0.1M HCl added and vortexed for 2 minutes; following centrifugation at 3000 rpm for 5 minutes, the aqueous layer was transferred to another tube.

- At pH 1 (upon addition of HCl), the free amines dissolved in the organic layer are converted into the hydrochloride salts which are soluble in the aqueous solution, while contaminants that are not soluble in water remain in the organic layer.

Phase 3

NaOH (0.5M, 2 mL) was added together with 3 mL of butyl chloride; vortex mixing followed for 2 minutes and then centrifugation for 5 minutes at 3000 rpm; the organic layer was removed and evaporated using Caliper Sciences Turbo Vap® LV at 50°C for 20 minutes.

- At pH 13.7 (upon addition of NaOH), amitriptyline and nortriptyline hydrochlorides are converted back into the free amines and extracted into the organic solvent, eliminating residual water-soluble contaminants. The butyl ether solvent evaporates, leaving a pure free amines residue.

Protein precipitation consisted of the following steps: 25 µL of clomipramine (100 µg/mL internal standard solution) were added to 0.5 mL serum spiked with 25 µL standard solution; vortex mixing was carried out for 3 minutes using a Vortex Genie 2® G560E; 1 mL acetonitrile was added to the solution using a glass syringe and vortex mixed for another 5 minutes; centrifugation followed using Eppendorf® Centrifuge 5414 (Eppendorf, Germany) at 10,000 rpm for 10 minutes; the supernatant was poured into glass tubes to evaporate to dryness in Caliper Sciences Turbo Vap® LV for 20 minutes, with the water bath set at 50°C. The protein precipitation protocol was amended to assess improvement in recovery of amitriptyline and metabolites by the addition of a drop of phosphate buffer (pH 5.6, 9 and 10 tested) or 0.5M NaOH to the samples at start.

The dried residues obtained following the sample preparation procedures were reconstituted in 400 µL of mobile phase, vortex mixed for 3 minutes and centrifuged at 10,000 rpm for 5 minutes prior to injection in the HPLC unit for analysis. Samples reconstituted in 200 µL of mobile phase were also tested, in attempt of increasing concentration and corresponding detection. In parallel, a search for laboratories that investigate blood concentrations of amitriptyline, nortriptyline and the hydroxy-metabolites did not direct to any potential providers. Quotations were obtained from three laboratories for the service of determining concentrations of amitriptyline, nortriptyline, and total, in serum samples. The logistics were complicated, and costs elevated, by the controlled temperature requirements during transportation. Through visiting The Doctors Laboratory in London, analysis of the samples for amitriptyline and nortriptyline serum levels by LC-MS was co-ordinated. RECIPE Chemicals + Instruments GmbH (Germany) were contacted to enquire about therapeutic drug monitoring platform kits for LS-MS/MS technologies. The available systems do not encompass the hydroxy-metabolites, implying that development of a new method was entailed.

2.3.4 LC-MS/MS method development

Deuterated standards and LC-MS grade solvents were sourced to generate multiple reaction monitoring (MRMs) for amitriptyline, nortriptyline and the hydroxy-metabolites, separation of parent drug and metabolites, and quantitative serum analysis, using an LC-MS/MS system at the Toxicology Laboratory, Mater Dei Hospital. Method development involved optimisation phases related to: the MS/MS signal parameters, the chromatographic separation for the isobaric metabolites which necessitated physical separation on the column, and sample extraction procedure with validation.

LC-MS grade water, ammonium formate, formic acid and methanol were procured from Carl Roth (Karlsruhe, Germany). Amitriptyline, nortriptyline, Z-10-hydroxynortriptyline, E-10-hydroxynortriptyline, Z-10-hydroxyamitriptyline, amitriptyline-D6 and nortriptyline-D3 were purchased from Clearsynth Labs Ltd. (Mumbai, India). E-10-hydroxyamitriptyline was acquired from Sigma-Aldrich (Missouri, USA). Standards purity ranged between 96.50% and 99.97%. Utak and Siemens supplied QC material. Serum samples, obtained from Mater Dei Hospital (Msida, Malta), were pooled for spiking when no traces of the analytes of interest were detected while separate serum samples were utilized for matrix interference studies.

The chromatographic system was composed of two Shimadzu Nexera (Kyoto, Japan) LC-30AD pumps with SIL-30AC auto-sampler, DGU-20A degasser, CTO-20AC oven and a CBM-20A controller. Chromatographic separation was achieved on an ACE-3 C18 column (50 x 2.1mm, 3µm) fitted with ACE Excel UHPLC pre-column filter (0.5µm) from Advanced Chromatography Technology Ltd (Aberdeen, Scotland). Column temperature was 40 °C throughout analysis and sample extracts were kept at 4 °C in auto-sampler. A gradient of 0.1% formic acid/10mM ammonium formate in water (mobile

phase A) and 0.1% formic acid/10mM ammonium formate in methanol (mobile phase B) at a flow rate of 0.25 mL/min was used. Gradient conditions were 45% mobile phase B at start, increasing to 90% mobile phase B at 3.5 min, held for 0.5 min and returned to 45% to equilibrate for 1 min. The total run time was of 5.5 min, with a 50% methanol 5 s rinse done before and after sample injection.

Positive electrospray ionization was employed on Shimadzu LCMS-8050 triple quadrupole mass spectrometer equipped with a DUIS-8050 dual ionisation unit. General parameter settings were set according to the tuning file (from auto mass calibration) with default settings for nebulising gas flow (3 L/min), heating gas flow (10 L/min), drying gas flow (10 L/min), CID gas (argon at 270kPa), interface temperature (300°C), desolvation line temperature (250°C) and heat block temperature (400°C). Multiple reaction monitoring (MRM) parameters were set via direct injections using LabSolutions software (v.5.93) to optimize for precursor and product ions as well as voltage selection for Q1 pre bias, CE and Q3 pre bias settings.

The stock solutions were prepared from pre-weighed purchased standards (and internal standards) in vials. The standards were separately dissolved in methanol and made up to 10 mL (20 mL for E-10-hydroxyamitriptyline) in volumetric flasks. An intermediate standard solution (5.0 µg/mL) containing all analytes (except internal standards) was prepared by dilution with methanol from stock solutions using Hamilton Microliter™ syringes in a 5.0 mL volumetric flask. Calibration standard solutions containing 1.0 to 400 ng/mL were prepared by spiking pooled serum samples using the intermediate stock in volumetric flasks. The lower concentration standard (1.0 ng/mL) was used to prepare the lower concentrations by serial dilution with pooled serum to the required concentration (0.1, 0.2, 0.5, 0.8 ng/mL). The calibration standards were allowed to

equilibrate overnight at 4°C after which the standards were aliquoted (300 µL) into microvials and stored at -30°C until used. Calibration was performed with freshly thawed standards as required. The working internal standard solutions were prepared separately by diluting the stock solutions in 20 mL volumetric flasks to a concentration of 200 ng/mL in methanol and stored at -20°C. The standards used for recovery and matrix effect studies were prepared on the day of study by spiked pooled serum (recovery) or individual samples (matrix effect) on the day of study.

For sample preparation, the isotopically labelled standards, at a concentration of 200 ng/mL in methanol, were used as internal standards (IS). A 20 µL aliquot of IS was added to 100 µL of sample, standard or control. The mixture was vortexed for 10 s and 200 µL of acetonitrile (cooled at -20°C) added to precipitate proteins. The mixture was again vortexed for 30 s and centrifuged for 5 min at 15,600 g. A volume of 50 µL supernatant was transferred to another micro-centrifuge tube and diluted with 150 µL of 0.1% formic acid/5mM ammonium formate. The mixture was transferred to an injection vial and 5 µL were injected into the LC-MS/MS system.

Recommendations in bioanalytical method validation guidelines[37,38,39] were followed to validate the method for precision and accuracy, specificy, linearity, lower and higher limits of detection, recovery, matrix effects and stability. Calibration standards were prepared from a pool of human serum spiked with all the analytes, except the deuterated IS. A set of 6 standards was prepared and calibration curves constructed with all analytes

[37] Food and Drug Administration. Bioanalytical method validation guidance for industry [Online]. FDA; US: 2018 [accessed 2019 Jul 28]. Available from: https://www.fda.gov/media/70858/download.

[38] European Medicines Agency. Guideline on bioanalytical method validation [Online]. EMA: 2011 [accessed 2019 Jul 28]. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf.

[39] ICH. ICH Harmonized Tripartite Guideline Q2(R1): Validation of Analytical Procedures: Text and Methodology [Online]. ICH: 2005 [accessed 2019 Jul 28]. Available from: https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf.

at serum levels of 0.5, 2, 10, 50, 150 and 400 ng/mL using area ratios of the quantification transition ions (analytes to respective IS) and plotting using linear regression with 1/x weighting without forcing through zero, achieving an r^2 (coefficient of determination) value >0.99 for all analytes. Recovery was determined at LLOQ and ULOQ by comparing peak area ratios (analyte/IS) between the sera spiked before and after extraction.

Matrix interference (ion suppression or enhancement) was investigated in six random serum samples. The samples were first processed as blanks and then as extracts spiked with standard at near LLOQ and ULOQ concentrations. The matrix factor (MF) was calculated by comparing peak areas of the analytes (including IS) from matrix, with peak areas of the analytes (of the same concentration) spiked in water. The IS normalised MF was calculated by dividing the MF of the analytes with the MF of the IS. The acceptance criteria for IS normalised MF were <15% (percent coefficient of variation, CV%) for concentrations at low and high level.

Intra-day precision (CV%) and accuracy (as bias) were calculated via six intra-day repetitions for all analytes at the LLOQ 0.5 ng/mL, 2.0 ng/mL, 10 ng/mL, 50 ng/mL and ULOQ 400 ng/mL from the six serum extracts. Inter-day precision and accuracy were similarly calculated after the same levels were analysed daily for 6 days. The acceptance criteria for intra-day and inter-day precision and accuracy were 15% for nominal concentrations, except 20% at LLOQ. LLOQ was established with signal-to-noise ratio (S/N) >5 and LOD was calculated on the S/N >3. Carry-over was investigated in the six sera by injection of a blank solution/sample after ULOQ standard. A 5 s wash of the autosampler needle with 50% methanol was performed before and after aspiration.

To assess stability, spiked controls, stored at -30°C were analysed once a week and relative response (peak area of analyte/peak area of IS) compared with spiked controls from the same batch analysed soon after preparation. Commercial controls, containing amitriptyline and nortriptyline, were analysed with the other controls once a week. The commercial controls were kept at 4 °C as per manufacturers' recommendation for the duration of the project. Quality control (QC) material for amitriptyline and nortriptyline was used at the mid- to high- range while for the lower concentration ranges and in the case of the hydroxy-metabolites, spiked samples were used as QC material. The LC-MS/MS method developed was considered for prospective application in the analysis of serum samples obtained from patients on amitriptyline therapy, with the clinical protocol of this research also involving genotyping patients for *CYP2C19* and *CYP2D6*.

2.3.5 Genomic DNA extraction and CYP450 genotyping

Peripheral blood is the customary source of genomic DNA and is regarded as the comparative standard (Hansen et al, 2007). Yields of DNA obtained from less conventional sources, such as buccal cells, may be altered by the technique and number of cells captured (Mulot et al, 2005). In the experimentation phase of this study, random buccal swabs and blood samples were used to develop a protocol for extracting DNA, and comparing outcomes in the subsequent genotyping stages. This process was carried out at the BioDNA Laboratory, Malta Life Sciences Park, after signing confidentiality documentation and providing buccal sample for record of own DNA profile. Detailed description of the procedures undertaken is documented in Appendix B, with handling taking place in the Microflow Advanced Bio-Safety Cabinet, and controls included in each technique.

For quick estimation of DNA yield and concentration, agarose gel electrophoresis was carried out. The preliminary indications were not encouraging and technical support was sought. Amelioration of buccal cells collection through repeated brushing was recommended, together with soaking and twisting of the swab until the solution becomes cloudy during DNA extraction. Longer incubating time should increase the DNA yield although over 3 µL DNA may inhibit PCR amplification. Incomplete inactivation of the proteinase K enzyme may also cause PCR failure. Amendments to the procedure for extracting DNA from buccal swabs included longer incubating times (from 5 minutes to 30 minutes), increasing heat inactivation time of proteinase K (from 3 minutes to 20 minutes), and using 2 µL of DNA for the PCR-based genotyping.

Experimentation with genotyping ensued, using Mutector^{TM} II reagents, procured from TrimGen Genetic Diagnostics, Nevada, US, and stored at –20^{o}C. The format of every kit comprises two assays with each tube designed to detect and differentiate a specific set of alleles, as outlined in Table 2-1, which provides a one-dimensional summary whereby 2850C>T and 4180G>C, for instance, may also appear in other variants. As an example, *CYP2D6* 1846G>A indicates that at the 1846 nucleotide position on the *CYP2D6* gene, the nucleotide G is replaced by a variant A nucleotide. This results in a non-functional protein caused by a splicing defect, which is responsible for the majority of poor metabolisers found in Caucasian populations (Owen et al, 2009). While with normal copy number there is no functional change for alleles **2A* and **2B*, *CYP2D6* activity is enhanced when their copy number increases.

Table 2-1: Single Nucleotide Polymorphisms detected by Mutector™ kits

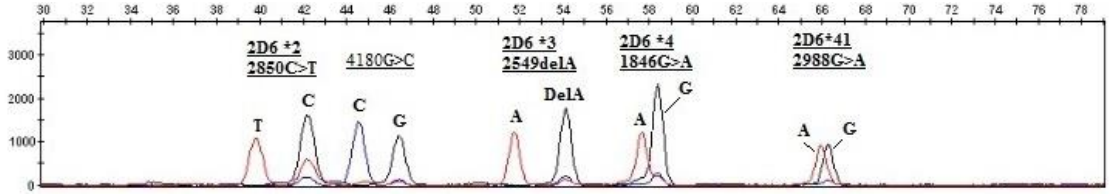
CYP2D6 Mutector™ Kit				*CYP2C19* Mutector™ Kit			
Tube	**Allele**	**Change**	**Activity**	**Tube**	**Allele**	**Change**	**Activity**
A	**2A*	*4180G>C*	Normal/Increased	A	**2*	*c.681G>A*	None
	**2B*	*2850C>T*	Normal/Increased		**3*	*c.636G>A*	None
	**3*	*2549delA*	None		**4*	*c.1A>G*	None
	**4*	*1846G>A*	None		**5*	*c.1297C>T*	None
	**41*	*2988G>A*	Decreased				
B	**6*	*1707delT*	None	B	**6*	*c.395G>A*	None
	**9*	*2615-17delAAG*	Decreased		**7*	*IVS5+2T>A*	None
	**10*	*100C>T*	Decreased		**8*	*c.358T>C*	Decreased
	**17*	*1023C>T*	Decreased		**17*	*c.-806C>T*	Increased

The analytical procedure carried out at the BioDNA Laboratory was analogous for both genes under study, following the protocol outlined in the respective user manual[40] - PCR amplification, PCR product clean-up, Genotyping reaction, Sample loading - as described in Appendix B. All reagents were thawed prior to use and negative and positive controls included for each run. Data analysis was carried out using the Gene Mapper ID v.3.2 software (Thermo Fisher Scientific, US). The individual SNPs are identifiable by peak colour and size, as per the reference of positive results[41] presented in Figure 2-5. *CYP2D6* gene deletion (**5*) shows as no peaks (homozygous) or lower peaks (heterozygous). Figure 2-6 shows an example from Trimgen[42], as a guide for understanding potential outcomes of *CYP2D6* Tube A. Analysis of this data should allow allele confirmation with the elimination of pseudo genes, which due to their similarity, could make the assay more complicated. The determined diplotype with an established enzyme activity score corresponds to a genotype which characterizes the phenotype. Should no variant SNP's be detected, the subject is presumed normal.

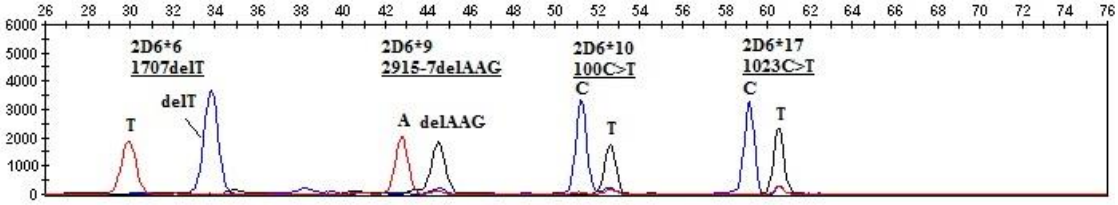
[40] TrimGen Genetic Diagnostics. Mutector™ Mutation Detection Kit [Online]. *CYP2D6* / *CYP2C19* Genotyping Reagents User Manual [accessed 2019 May 1]. Available from: https://www.trimgen.com/pharmacogenetics-genotyping.
[41] TrimGen Genetic Diagnostics. Mutector™ Mutation Detection Kit [Online]. *CYP2D6* Genotyping; and *CYP2C19* Genotyping [accessed 2019 May 1]. Available from: https://www.trimgen.com/products/CYP2D6-Genotyping; and https://www.trimgen.com/CYP2C19-Genotyping.
[42] TrimGen Genetic Diagnostics. Mutector™ Mutation Detection Kit. *CYP2D6* Genotyping [as above].

Figure 2-5: Sample results for *CYP2D6* and *CYP2C19* genotyping with Mutector™ kits (Trimgen)

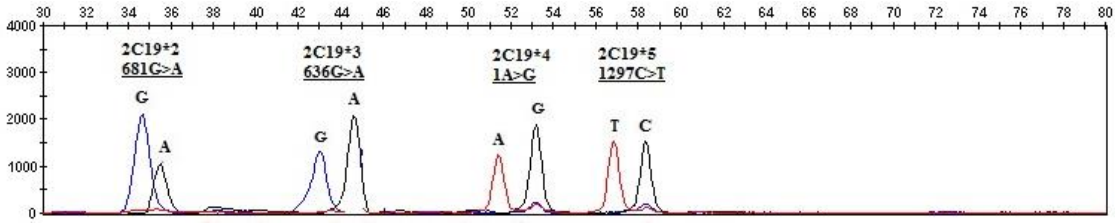
CYP2D6 Mutector™ Kit Tube A detects 5 variations:



CYP2D6 Mutector™ Kit Tube B detects 4 variations:



CYP2C19 Mutector™ Kit Tube A detects 4 variations:



CYP2C19 Mutector™ Kit Tube B detects 4 variations:

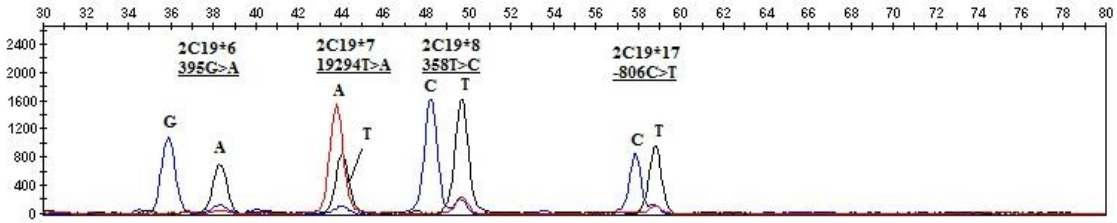
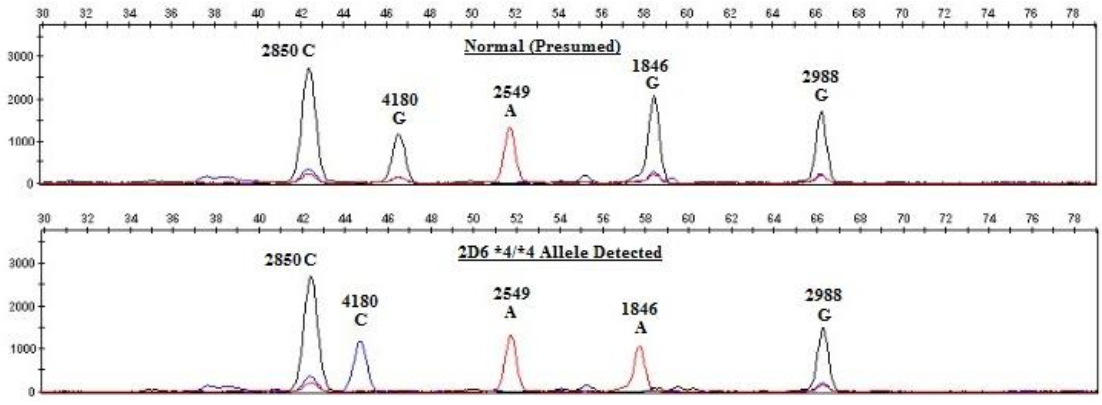


Figure 2-6: *CYP2D6* Tube A genotyping results (Trimgen)



The outcomes of experimentation with the Mutector™ kits were discussed with the technical personnel at TrimGen Genetic Diagnostics, US. Duplicate labelled buccal swabs were sent to their CLIA-certified laboratory, X-Gene Diagnostics, in order to also consider Copy Number Variation analysis. The specimens were analysed through the validated method, developed by X-Gene Inc, using real-time PCR with TaqMan® SNP Genotyping (Thermo Fisher). The laboratory reports >99% analytical specificity and sensitivity for detection of the following variants: *CYP2C19 - *2, *3, *4, *4B, *5, *6, *7, *8, *9, *10, *17*; *CYP2D6 - *2, *3, *4, *4M, *6, *7, *8, *9, *10, *12, *17, *29, *35, *41, *5* (gene deletion), and *xN* (gene duplication). Performance characteristics of the assays were validated following the 1988 CLIA standards, by X-Gene Diagnostics (CLIA No. 21D2093389, Permit No. 2257), with the testing being performed at the laboratory in Frederick, MD State.

2.4 Clinical investigation

The systematic consideration of analytical and technical requisites for the practical application of a pharmacogenetic approach supported the research in moving forward to the clinical scenario. Membership in the Clinical Pharmacogenetics Implementation Consortium (CPIC), permitted involvement in regular conference calls, discussions with pertinent members, particularly Dr Charity Nofziger, as well as access to documents under development, aimed to translate genetic results into actionable prescribing decisions for specific drugs, including tricyclic antidepressants. The insight gained though participation in this pharmacist-led initiative facilitated the planning of the clinical methodology. Further understanding and experience was acquired by completing the American College of Clinical Pharmacy (ACCP) Academy Programme – Precision Medicine: Applied Pharmacogenomics – between September and November 2018. The

programme was designed to help clinical pharmacists learn empirical strategies for integrating precision medication services for current patient populations, through the study of practice-based clinical cases involving psychiatry and pain. Through online graded modules and a live workshop held in October 2018 during ACCP's Global Conference on Clinical Pharmacy in Seattle, Washington, a pharmacogenomics implementation plan was developed, including assessment of the strengths, weaknesses, opportunities and threats identified with respect to application in a clinical setting.

2.4.1 Scope and practice setting

The clinical aspect of the research intended to explore whether genotyping for relevant CYP polymorphisms is apt to translate these genetic biomarkers into assets for delivering individualised treatment with minimal risk and whether amitriptyline may serve as an example for the application of precision pharmacotherapy in the case of established drugs. The scope was to evaluate the pragmatic implications that genotype-guided dosing recommendations may have on amitriptyline therapy, considering the potential of phenoconversion, and to assess how metaboliser status and dose-related reference ranges may facilitate interpretation of therapeutic drug monitoring. Psychiatric and pain management settings were identified to study the interplay between genotype, blood levels and clinical outcomes.

Mater Dei Hospital, the general and teaching hospital located in Msida, Malta, was identified as setting for the clinical investigation, since it embodied the multidisciplinary structure required by the research design. Institutional approvals for access to subjects and data, as well as approval from persons directly responsible for subjects were obtained, including endorsement by: CEO, Mater Dei Hospital (MDH); Data Protection Officer,

MDH; Chairperson, Department of Psychiatry, MDH; Chairperson, Department of Anaesthesia, MDH; Chairperson, Department of Medicine, MDH; Chairperson, Department of Pathology, MDH; Chairperson, Department of Cardiology, MDH; CEO, Mount Carmel Hospital (MCH); Data Protection Officer, MCH and Mental Health Services.

The identification, consultation, enrolment and follow-up of patients was supported by Consultant Psychiatrists holding clinics at Mater Dei Hospital Psychiatric Out-Patients and a Consultant Anaesthetist within the Pain Clinic of Mater Dei Hospital. Through collaboration with the Department of Pathology and the ICT Department of Mater Dei Hospital, an electronic profile - 'Amitriptyline Order Set' - was created in the system. Access was provided to the clinicians involved in the project, enabling them to order renal and hepatic function tests as well as an electrocardiographic examination (ECG) for the identified patients who consented to participation in this research.

2.4.2 Ethical considerations

Approval for this research was granted by the University Research Ethics Committee, University of Malta (Appendix C) in July 2017. Amendments were subsequently submitted and approved (October 2017), to expand the study rationale of the protocol endorsing the recruitment of psychiatric patients (Ref. 23/2017), to also consider a cohort of patients receiving amitriptyline for neuropathic pain.

The principles of research ethics, embedded in the Declaration of Helsinki and the Nuremberg Code, emphasize the importance of informed voluntary consent. Ethical considerations apply to research in any specialty, but issues such as capacity and consent in mental health studies draw higher scrutiny, owing to the potential impact of psychiatric

disorders on cognitive functioning (Cooper et al, 2016, Carrier et al, 2017). While independent review of protocols assesses scientific validity and ethical adequacy of the means, informed consent follows the individual's decision about research participation. If the proposed research is contrary to patient interest, it would not gain ethical approval. Nonetheless, good practice entails the assurance that the person is capable of understanding, retaining and weighing up information and communicating their decision. The exclusion of participants who do not meet the latter criteria may deprive them access to the opportunity of active participation in innovative research.

The NHS Health Research Authority[43] states that, as per the UK Mental Capacity Act 2005, unless established otherwise, capacity should be assumed. Back in 2005, Rikkert and colleagues highlighted how research ethics committees may differ in their interpretation of clinical studies across the EU, with substantial heterogeneity in judgements on capacity to consent and acquisition of informed consent. The impairment that mental disorders may pose on decision making, may not adversely affect a subject's capacity, especially in the early phases, albeit additional care may be warranted in the informed consent process.

In line with the Mental Health Act (Chapter 525 of the Laws of Malta)[44], an Independent Specialist was appointed by the Commissioner for Mental Health, to examine individuals identified through the Psychiatric Out-Patients and certify their capacity to give free and informed consent and that the expected benefits of the research are likely to outweigh any potential harm.

[43] NHS Health Research Authority. Questions and Answers – Mental Capacity Act 2005 [Online]. NHS Health Research Authority; 2013 [accessed 2019 May 1]. Available from: http://www.hra.nhs.uk/resources/research-legislation-and-governance/questions-andanswers-mental-capacity-act-2005.

[44] Mental Health Act (Cap 525). Laws of Malta [Online]. Malta; 2012 [accessed 2019 May 1]. Available from: http://www.justiceservices.gov.mt/DownloadDocument.aspx?app=lom&itemid=11962&l=1.

2.4.3 Recruitment protocol

The research proposal granted with ethics approval was endorsed by eight psychiatrists and one pain management consultant. The inclusion criteria for prospective patient participation in the research were:

i. Over 18 years of age
ii. Capable of giving free and informed consent
iii. Under the care of the specific consultant firms
iv. Attending the Psychiatric Outpatient Clinic or Pain Clinic at Mater Dei Hospital
v. Medical records available, including contact details
vi. Pain or depressive illness being treated with amitriptyline as monotherapy or add-on.

Patients who did not meet the inclusion criteria were excluded accordingly.

An internal database listing all individuals entitled to amitriptyline under the Pharmacy of Your Choice Scheme (POYC) scheme was obtained. The number of patients entitled to amitriptyline through the public health sector in Malta amounts to 2847, as per the data extracted from the POYC database in October 2017. Entitlement records list one thousand and ninety-two (1092) patients for 10mg amitriptyline tablets, while one thousand, seven hundred and fifty-five patients (1755) are registered for the 25mg tablets. The Identity Card numbers recorded in the extensive list were subsequently searched within the electronic CPAS Patient Interface at the Psychiatric Outpatients (POP) of Mater Dei Hospital, to identify the patients that had an upcoming appointment with one of the participating consultant psychiatrists at the POP Clinic during Q1 and Q2 2018 (Figure 2-7).

Figure 2-7: Recruitment of patients on amitriptyline, under psychiatric care

Number of patients	Context and involvement
2847	**Listed as being on amitriptyline (Malta public health sector)**
276	**Under the care of one of the participating Consultant Psychiatrists, having a Psychiatric Outpatients (POP) appointment in 2018**
159	**Scheduled by POP for appointment between January and June 2018**
75	**Attended appointment and were briefed on this research by clinician**
42	**Contacted by investigator for prospective patient involvement**
36	**Scheduled by investigator for appointment with Independent Specialist**
24	**Attended appointment and proceeded within this research**

Two hundred seventy-six (276) of these individuals were being followed by one of the eight Consultant Psychiatrists involved in the research and had an appointment scheduled at the Psychiatric Out-Patients Clinic of Mater Dei Hospital for 2018. The one hundred fifty-nine (159) appointments scheduled for dates between January and June 2018 were considered. Through liaison with the nursing staff, the Information Sheet (English/Maltese, Appendix D) was affixed to the hospital files of the identified patients, on the day of their POP appointment. The consultants, or clinicians within their firm, discussed the proposed research with the prospective participants to identify patients that were interested to learn more about the study. Seventy-five (75) patients attended for the POP appointment and were briefed by the attending clinician. The number of prospective participants dropped from 159 to 75 due to a number of appointments being cancelled, appointments being rescheduled to clinics outside hospital, changes in the consultant responsible for the individual, patients not attending for their scheduled appointment and deaths.

A number of patients were excluded because their hospital files were not made available, notes indicated that amitriptyline had been withdrawn or records were incomplete, missing any contact numbers. Forty-two (42) patients were contacted to schedule a meeting with the Independent Specialist. One patient had switched consultant, one stopped amitriptyline, one refused, and three had evident difficulty to understand and communicate. Thirty-six (36) patients, 15 males and 21 females, agreed to meet the Independent Specialist and were convened for this consultation on one of two dates: 26 September 2018 and 10 October 2018. The Independent Specialist, a consultant psychiatrist with over 30 years' experience, performed individual assessment of each of the twenty-four (24) patients that eventually attended for the meeting. Once the protocol was explained to the subjects and capacity certified, patients were invited to make an informed decision and consider signing the consent form (English/Maltese, Appendix E). All 24 patients proceeded in the study, following written informed consent.

Recruitment of patients from pain management commenced in parallel, as of Q2 2018. Patients attending the Pain Clinic at Mater Dei Hospital for suspected neuropathic pain, seen by the consultant anaesthetist involved in the project, were considered, if the treatment plan included amitriptyline. The clinician explained the rationale of the research (as per Information Sheet in Appendix D) to the identified patients. If interested to participate, these individuals were eventually contacted for consent (Appendix E) and involvement, according to the research protocol. Twenty (20) patients were recruited from the Mater Dei Pain Clinic, between May and November 2018.

In total, a cohort of 44 out-patients of any gender, over 18 years of age, whose treatment included the tricyclic antidepressant amitriptyline, were recruited from Mater Dei Hospital by purposive convenience sampling. The study population comprises patients

attending the pain clinic or the psychiatric outpatient clinic for pain or depressive illness sufficiently severe to require therapy, with amitriptyline as monotherapy or as add-on, and follow up through the state hospital outpatients' services. The consent procedure, data collection and sample collection were carried out on the same day, whenever possible, in attempt of limiting patient discomfort.

2.4.4 Sample collection

Tricyclic antidepressants can be monitored using plasma or serum, with the latter allowing greater ease of extraction and no fibrin clots are involved (Mitchell, 2000). Blood samples for serum measurements were taken in the morning. It was recommended that patients postpone their morning dose of amitriptyline until after the samples were collected, if applicable. For any cases of recent amitriptyline therapy onset, blood withdrawal was scheduled following continual dosing and achievement of steady-state (Linder & Keck, 1998). The amitriptyline dose and time since the last dose administration were noted upon blood sample collection to enable calculations for circulating concentrations. Blood withdrawal took place at the Pathology Department of Mater Dei Hospital, through liaison with the responsible phlebotomist. One blood sample per patient was submitted for the routine renal and liver function tests, the results of which were made available within a few working days.

Blood samples for concentration analysis were collected in red-stopper tubes. Separation of serum from other cellular components was executed without delay. The filled red top blood collection tubes were allowed to sit upright at room temperature for 30 minutes for clot formation. The blood samples were then centrifuged at 3000 g for 10 minutes at room temperature in centrifuge Z446K (HERMLE Labortechnik GmbH, Germany). A

pipette was used to transfer 500 µL serum aliquots into labelled vials which were stored in a -30˚C freezer at the Toxicology Laboratory, Mater Dei Hospital.

To provide for *CYP2D6* and *CYP2C19* genotypic measurements, buccal cells were collected, as per the following procedure:

i. Swabs, two per patient, removed from the sterile tube, one at a time, to use one swab for each cheek.

ii. The inside of each cheek firmly brushed up and down ten times, rotating to cover the surface of the swab head; repeated for the other cheek with the second swab.

iii. Swabs dried for 10 minutes before closing in labelled tube.

Buccal swabs were stored at room temperature until analysis. DNA stabilising solutions or preservative agents were not added to any of the samples collected, although in retrospect, such considerations could have been worthwhile seeing that instant analysis was not feasible (Swinfield et al, 2009). Whole blood samples of 3mL collected in EDTA purple-top tubes, were stored refrigerated for potential DNA extraction, should the genotyping procedure through buccal cells fail. Prolonged storage at 4˚C may affect DNA yield, but the impact on DNA quality is expected to be minimal (Richardson et al, 2006, Bulla et al, 2016).

2.4.5 Patient assessment

Hospital files pertaining to patients identified for the research were accessed through the Medical Records Department at Mater Dei Hospital. The data collection forms (Appendix F) were completed through review of the available clinician notes and communication with the patient upon meeting, whereby the subjects' contribution was also necessitated for scoring rating scales, as described hereafter.

In light of the heterogeneous symptomatology between depressed patients, no gold standard has been identified to measure depressive symptoms, although a number of different scales may be used (Hodgson, 2014). Work by Uher et al (2008) explored three scales, one self-reported - the Beck Depression Inventory (BDI; Beck et al, 1961), and two clinician-rated – the Hamilton Depression Rating Scale (HDRS-17; Hamilton, 1960, 1967) and the Montgomery-Asberg Depression Rating Scale (MADRS; Montgomery & Asberg, 1979). The authors concluded that MADRS outperforms the other scales for accuracy and inter-rater reliability of detecting depression symptoms.

The MADRS 10-item scale is principally receptive to the effects of antidepressants in patients receiving treatment for major depression (Roffman et al, 2010). Permission by the author is granted for the use of the MADRS scale by clinicians in their practice and by researchers in non-industry studies. MADRS was employed for the assessment of patients in the psychiatric setting of this study as a measure of illness severity in terms of the type and magnitude of symptom burden present. MADRS addresses the following symptom domains: mood (sadness apparent/reported, loss of interest, suicidal ideation), anxiety (tension), appetite (reduced appetite), sleep (insomnia), functional status (difficulty in activities), ability to think (concentration), and general psychiatric distress (pessimism), through a fixed scaling of seven points (from 0 through 6).

For the pain research setting, permission was obtained from Pfizer Limited to use painDETECT. Freynhagen et al (2006), developed and validated the painDETECT questionnaire (PD-Q) in lower-back pain patients, through co-operation with the German Research Network on Neuropathic Pain. The gradation of pain is scored from 0 to 5 (not at all = 0, hardly noticed = 1, slightly = 2; moderately = 3, strongly = 4, very strongly = 5). Scores of -1 to 2 are added as per the pain course pattern and pain radiation reported.

The final score is between –1 and 38, with higher scores, particularly over 19, indicating greater likelihood of a neuropathic pain component. Robust psychometric evidence is reported on the validity and reliability of painDETECT for distinguishing average pain severity in neuropathic pain patients (Cappelleri at el, 2014). The questionnaire has been investigated in rheumatoid arthritis and osteoarthritis, thoracotomy, tumor diseases, fibromyalgia, diverse musculoskeletal conditions and diverse other conditions, including studies on the effect of drug treatment of patients with a neuropathic pain component (Freynhagen et al, 2016). Treatment effects may be denoted through pain-severity levels, indicating outcomes on pain symptoms (Sadosky et al, 2016).

Both MADRS and painDETECT were rated on one occasion by the recruited patients, and the measures were not intended to make inferences about response to amitriptyline therapy but rather to assess the present-day status of the respective conditions. Side-effects were also rated once by the participants. As part of the GENDEP project, side effects that had been previously associated with antidepressants have been specifically identified in designing the self-report ASEC - Antidepressant Side Effect Checklist (Uher et al, 2009), including: dry mouth, drowsiness, insomnia, blurred vision, headache, constipation, diarrhoea, increased appetite, decreased appetite, nausea or vomiting, problems with urination, problems with sexual function, palpitations, feeling light-headed on standing, feeling like the room is spinning, sweating increased, body temperature, tremor, disorientation, yawning, and weight gain. A good correlation was reported between the comprehensive interviewer-rated UKU - Udvalg for Kliniske Undersoegelser Side Effects Rating Scale (Lingjaerde et al, 1987) and ASEC (Hodgson, 2014). Permission to use ASEC in this project was granted by The Royal College of Psychiatrists, London, UK. All patients recruited in the research were guided to score the

21 items indexed in ASEC on a four-point scale (0 absent; 1 mild; 2 moderate; 3 severe), making note on whether the symptom is likely to be a side-effect of amitriptyline.

All patients underwent an electrocardiographic (ECG) examination, and each 12-lead ECG report was analyzed for heart rate, PR, QRSd, and QTc. Further to the publication by Taavola[45] and PRAC considerations of a signal of dry eye being associated to amitriptyline, patients recruited from POP were also asked to give a score for dry eye (0-3), if perceived to be related to the medication. It was explained that signs and symptoms, which usually affect both eyes, may include: a stinging, burning or scratchy sensation in the eyes; stringy mucus in or around the eyes; sensitivity to light; eye redness; a sensation of having something in the eyes; difficulty wearing contact lenses; difficulty with night-time driving; watery eyes, which is the body's response to the irritation of dry eyes; blurred vision or eye fatigue.

2.5 Data processing

IBM SPSS Statistics® software, version 25, was used for statistical analysis. Data distribution was tested for normality using the Shapiro-Wilk test. Amitriptyline and nortriptyline concentrations in 44 patient serum samples, measured individually by the LC-MS/MS method developed and validated in-house, and through LC-MS analysis outsourced to The Doctors Laboratory, were compared. The Spearman correlation coefficient, ranging from -1 to 1, indicated the strength of the relationship between the two continuous variables. The null hypothesis for this test is that there is no relationship between the concentrations of amitriptyline and nortriptyline measured by the two

[45] Taavola H. Amitriptyline and dry eyes – an ADR overlooked in labelling. WHO Pharmaceuticals Newsletter No.5. World Health Organization; 2017.

methods of analysis, and the alternative hypothesis is that a relationship exists between the in-house measurements and those reported by the out-sourced laboratory. A p-value less than the 0.05 level of significance implies that the relationship is significant and not attributed to chance. The non-parametric Wilcoxon signed ranks test was used for the paired data sets to assess whether the medians of the concentrations measured by the two analytical methods differ significantly. The clinical evaluations proceeded henceforth using the levels measured by the developed LC-MS/MS method.

Spearman correlation was used to assess the relationship between daily amitriptyline dose and the concentrations of amitriptyline, nortriptyline, and the hydroxy-metabolites, for 42 patients on amitriptyline therapy (2 cases out of the 44 patient serum measurements were excluded since the patients did not postpone the morning amitriptyline dose, meaning that the blood sample was withdrawn around 4 hours post-dose). Differences in concentrations related to age and gender were assessed.

For subsequent analysis, the following patients were excluded from the total 44 participants: the 2 subjects having inconclusive genotyping results, the 2 subjects having blood withdrawn at 4-hours post dose, and 7 subjects whose amitriptyline dosage regimen entailed unequal distribution of the daily dose at the different time intervals (such as higher dose at night). Since the distribution of concentrations was right-skewed and did not satisfy the normality assumption, generalized linear models were used to relate the nortriptyline to amitriptyline ratio, and the hydroxy-metabolites to parent ratios, to a number of predictors collectively. CYP2C19 and CYP2D6 metaboliser status and the risk of CYP inhibition by co-administered drugs were included as factors. The models assume a gamma distribution and an identity link function. Analysis of inter-patient variability in the measured concentrations of the hydroxy-metabolites proceeded through

dose-normalised Ct levels, with administration regime and time of blood withdrawal (in the range of 11-18 hours post-dose) construed to have minimal impact.

The 2017 update to the Consensus Guidelines for Therapeutic Drug Monitoring in Neuropsychopharmacology (Hiemke et al, 2018) was studied. Following discussions with authors, particularly Dr Christine Greiner and Prof Christoph Hiemke, who re-established the dose-related reference ranges for the latest guidance on therapeutic drug monitoring in psychiatry, the mathematical functions proposed in their work were adapted to facilitate interpretation of the outcomes in this research. The expected concentrations for amitriptyline and nortriptyline (Ct) were estimated using Equation I, where Dm is the maintenance dose, di is the dosing interval, CL/F is the apparent total clearance (used as reciprocal), ke is the elimination rate constant ($\ln 2/t_{½}$), and t is the time of blood withdrawal. The dose-related reference ranges were calculated, based on the dosage regimen and sampling time, for each individual patient to identify those whose measured concentrations were not within the predicted range. A working example follows.

Equation I: $$\boldsymbol{Ct = \left(\frac{Dm}{di}\right) x \left(\frac{F}{CL}\right) x \left[\frac{(ke\ x\ di)}{(1-e^{-ke\ x\ di})}\right] x \left(e^{-ke\ x\ t}\right)}$$

Example: *Patient on 10mg amitriptyline once daily; blood withdrawn 15 hours post-dose*

$$\text{Amitriptyline Ct} = \left(\frac{10}{24}\right) \text{x } \left(\frac{1}{62.58}\right) \text{x } \left[\frac{(0.0365 \text{ x } 24)}{(1 - e^{-0.0365 \text{ x } 24})}\right] \text{ x } (e^{-0.0365 \text{ x } 15}) = 5.78 \text{ ng/mL}$$

$$\text{Nortriptyline Ct} = \left(\frac{10}{24}\right) \text{x } \left(\frac{1}{86.1}\right) \text{x } \left[\frac{(0.0231 \text{ x } 24)}{(1 - e^{-0.0231 \text{ x } 24})}\right] \text{ x } (e^{-0.0231 \text{ x } 15}) = 4.45 \text{ ng/mL}$$

	Dose-related reference range		
Amitriptyline [Ct ± SD (5.78 x 0.289)]	4.11 ng/mL	to	7.45 ng/mL
Nortriptyline [Ct ± SD (4.45 x 0.424)]	2.56 ng/mL	to	6.34 ng/mL

The Kruskal Wallis test and logistic regression models were used to assess the influence of CYP2D6 and CYP2C19 metaboliser status, and concomitant CYP inhibitors, on the

dependent variable: measured Ct for amitriptyline + nortriptyline. In logistic regression models, with a logit link function, the pseudo r^2 value measures goodness of fit in ranges from 0 to 1 where a value close to 1 indicates a very good fit and a value close to 0 indicates a poor fit. A forward procedure was used to identify the parsimonious model which includes solely significant main effects.

A dose-related concentration (DRC) factor was next computed for the o.d., b.d., and t.d.s, scenarios with the time interval taken as 24, 12, and 8 hours respectively. This enabled estimation of the trough (Cmin) dose-related reference ranges for each individual patient. Equation II, where Ct is concentration at time t, tmin is the time at Cmin and ke is the elimination rate constant, was used to determine patient Cmin based on the measured concentrations at time of blood withdrawal. A working example follows.

Example: *Patient on 10mg amitriptyline once daily; blood withdrawn 15 hours post-dose*

$$\text{DRC amitriptyline} = \frac{\left(\frac{1}{62.58}\right) \text{x} \left[\frac{(0.0365 \text{ x } 24)}{(1 - e^{-0.0365 \text{ x} 24})}\right] \text{x } (e^{-0.0365 \text{ x } 24})}{24} = 4.2 \text{ x} 10^{-4}$$

$$\text{DRC nortriptyline} = \frac{\left(\frac{1}{86.1}\right) \text{x} \left[\frac{(0.0231 \text{ x } 24)}{(1 - e^{-0.0231 \text{ x} 24})}\right] \text{x } (e^{-0.0231 \text{ x } 24})}{24} = 3.6 \text{ x} 10^{-4}$$

				Dose-related reference range	
	Dose x DRC low			Dose x DRC high	
Amitriptyline [DRC ± SD (0.42 x 0.289)]	10 x 0.30	*3.0 ng/mL*	*to*	10 x 0.54	*5.4 ng/mL*
Nortriptyline [DRC ± SD (0.36 x 0.424)]	10 x 0.21	*2.1 ng/mL*	*to*	10 x 0.51	*5.1 ng/mL*
Amitriptyline + Nortriptyline	10 x 0.51	*5.1 ng/mL*	*to*	10 x 1.05	*10.5 ng/mL*

	Example Measured Ct	*Cmin determination using Equation II:* $\text{Cmin} = \text{Ct x } e^{-\text{ke}(\text{tmin}-\text{t})}$	
Amitriptyline	6 ng/mL	$\text{Cmin} = 6 \text{ x } e^{-0.0365(24-15)}$	*4.3 ng/mL*
Nortriptyline	4 ng/mL	$\text{Cmin} = 4 \text{ x } e^{-0.0231(24-15)}$	*3.2 ng/mL*
Amitriptyline + Nortriptyline	10 ng/mL		*7.5 ng/mL*

This exercise identified the patients whose Cmin for amitriptyline, nortriptyline, and amitriptyline + nortriptyline was outside the predicted range. Cmin levels were

normalised to the dosing schedule to allow inter-patient comparisons in subsequent analyses. The Kruskal Wallis test was used to assess the influence of CYP2D6 and CYP2C19 metaboliser status, and concomitant CYP inhibitors, on the normalised amitriptyline, nortriptyline and amitriptyline + nortriptyline Cmin concentrations. A generalized linear model was successively employed since the dependent variable was no longer categorical (below/within/above range) but continuous (normalised Cmin).

The analyses were latterly repeated, replacing the lab-reported CYP2D6 metaboliser status with the *CYP2D6* activity score (as continuous variable/covariate) or an 'updated' CYP2D6 metaboliser status (as categorical variable/factor), in line with the latest CPIC consensus for genotype to phenotype, i.e. the 'normal metaboliser status' of patients with the following diplotypes - **1/*3, *1/*4, *2A/*4, *10/*10* - was switched to 'intermediate metaboliser status'. Genotype-guided dosing recommendations were assessed for the 17 psychiatry patients in the 33-patient cohort, informed by the CPIC interpretation guidance and the data analysis carried out.

Preliminary assessment of the side-effect measures was conducted during data collection, on a group of 13 patients from each of the two recruiting arms (n=26). The pilot investigation was intended to study ECG parameters of relevance, particularly QT-correction, and anticipated frequencies for the 21 effects listed in the Antidepressant Side Effect Checklist (ASEC). Upon recruitment of the entire 44-patient cohort, the analysis of side-effect outcomes proceeded, informed by the preliminary observations, with sample size varying subject to the practicable genotyping and serum levels data available for the participants, as explained. Statistical measures - Spearman, Mann Whitney, Kruskal Wallis and Chi squared tests - were used to analyse associations, according to the nature of the variables.

Kruskal Wallis was used to assess relationship between CYP2D6 and CYP2C19 metaboliser status, as determined by genotype, and total side-effect burden, and dry mouth or drowsiness scores (n=42). The Chi squared test was used to assess correlations between CYP parameters and ECG outcome as a categorical variable (normal/abnormal). The null hypothesis specifies that there is no association between two categorical variables and is accepted if the p-value exceeds the 0.05 level of significance. The alternative hypothesis specifies that there is an association between two categorical variables and is accepted if p-value is less than the 0.05 criterion.

The Mann Whitney test was used to investigate the relationship between electrocardiogram results and measured concentrations of amitriptyline, nortriptyline, Z-10-hydroxynortriptyline, E-10-hydroxynortriptyline, Z-10-hydroxyamitriptyline and E-10-hydroxyamitriptyline, at time of blood withdrawal. The analysis was repeated for total side-effect burden, dry mouth and drowsiness scores, against measured concentrations, using Spearman correlation (since variables are continuous). The Kruskal Wallis test was used to assess whether dry mouth score varied according to the patient's amitriptyline + nortriptyline concentration being below, within, or above the expected range (n=33). Associations related to dose and time since onset of therapy with amitriptyline were assessed, categorising patients as having been administered amitriptyline for (i) less than 12 months, or (ii) over 12 months. Logistic regression models were used to further investigate the side-effect outcomes.

Chapter 3
Results

3.1 Regulatory inferences

Preliminary study inferences were presented at the December 2016 virtual meeting of the European Medicines Agency (EMA) Pharmacogenomics Working Party (PgWP) following discussions with PgWP Chair, Dr Krishna Prasad, during a face-to-face meeting at the Medicines and Healthcare products Regulatory Agency (MHRA), London. This project was outlined at the April 2017 Strategic Review & Learning Meeting of the EMA Pharmacovigilance Risk Assessment Committee (PRAC) that monitors safety issues related to medicinal products, a number of which may have a potential pharmacogenomic link. Interactions with pertinent experts pursued via meetings, correspondence and teleconferences to engage in the regulatory developments on pharmacogenomics. The regulatory inferences and perspectives on translational pharmacogenetics emergent from this research, in relation to amitriptyline, were published[46].

3.1.1 Harmonisation of the amitriptyline Summary of Product Characteristics

European sources of medicinal product information, particularly decentralised SmPCs, and Japanese drug labeling, are reported to be less complete and applicable than US labels with respect to pharmacogenomic information (Shimazawa & Ikeda, 2013, Reis-Pardal et al, 2017). The FDA prescribing information (PI) lists all approved indications, routes of administration and known adverse effects. Results obtained from clinical trials as well as post-marketing surveillance are reflected in the summary of essential scientific and medical information known about the medicine.

[46] Mifsud Buhagiar L, et al. Regulatory sciences and translational pharmacogenetics: amitriptyline as a case in point. *Drug Metab Pers Ther* 2019;34(2). doi:10.1515/dmpt-2019-0005.

The official labelling for amitriptyline[47] presents the following text under 'Drug Interactions' as subsection of the 'Precautions' labelling section:

"*Drugs Metabolized by P450 2D6*

The biochemical activity of the drug metabolizing isozyme cytochrome P450 2D6 (debrisoquin hydroxylase) is reduced in a subset of the Caucasian population (about 7 to 10% of Caucasians are so called "poor metabolizers"); reliable estimates of the prevalence of reduced P450 2D6 isozyme activity among Asian, African and other populations are not yet available. Poor metabolizers have higher than expected plasma concentrations of tricyclic antidepressants (TCAs) when given usual doses. Depending on the fraction of drug metabolized by P450 2D6, the increase in plasma concentration may be small, or quite large (8 fold increase in plasma AUC of the TCA).

In addition, certain drugs inhibit the activity of this isozyme and make normal metabolizers resemble poor metabolizers. An individual who is stable on a given dose of TCA may become abruptly toxic when given one of these inhibiting drugs as concomitant therapy. The drugs that inhibit cytochrome P450 2D6 include some that are not metabolized by the enzyme (quinidine; cimetidine) and many that are substrates for P450 2D6 (many other antidepressants, phenothiazines, and the Type 1C antiarrhythmics propafenone and flecainide). While all the selective serotonin reuptake inhibitors (SSRIs), e.g., fluoxetine, sertraline, and paroxetine, inhibit P450 2D6, they may vary in the extent of inhibition. The extent to which SSRI-TCA interactions may pose clinical problems will depend on the degree of inhibition and the pharmacokinetics of the SSRI involved. Nevertheless, caution is indicated in the coadministration of TCAs with any of the SSRIs and also in switching from one class to the other. Of particular importance, sufficient time must elapse before initiating TCA treatment in a patient being withdrawn from fluoxetine, given the long half-life of the parent and active metabolite (at least 5 weeks may be necessary).

Concomitant use of tricyclic antidepressants with drugs that can inhibit cytochrome P450 2D6 may require lower doses than usually prescribed for either the tricyclic antidepressant or the other drug. Furthermore, whenever one of these other drugs is withdrawn from co-therapy, an increased dose of tricyclic antidepressant may be required. It is desirable to monitor TCA plasma levels whenever a TCA is going to be coadministered with another drug known to be an inhibitor of P450 2D6."

The prescribing information in the FDA-approved drug label does not quantify the recommended dose adjustments for CYP2D6 metabolisers and makes no direct reference to CYP2C19. Conversely, in the 'Interactions' section of the Japanese product information, there is reference to cytochrome P450 2D6 being mainly responsible for the

[47] U.S. National Library of Medicine. FDA Label: Amitriptyline hydrochrolide [Online]. DailyMed; NIH NLM [accessed 2018 August 26]. Available from: https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=1e6d2c80-fbc8-444e-bdd3-6a91fe1b95bd&audience=consumer.

metabolism of amitriptyline, together with CYP3A4, CYP2C19 and CYP1A2. Caution is recommended for use in combination with CYP2D6 inhibitors including fluoxetine, paroxetine, quinidine, propafenone, flecainide and cimetidine due to potential increase in blood concentration of amitriptyline and enhancement of its action.

Further to being approved in more than 56 countries worldwide, amitriptyline containing products have been marketed in Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Germany, Spain, Estonia, Finland, France, Greece, Hungary, Croatia, Iceland, Italy, Lithuania, Luxembourg, Latvia, Malta, Netherlands, Norway, Poland, Portugal, Sweden, Slovenia, Slovakia and the UK. Being authorised via national procedures, divergent national decisions taken by Member States have resulted in product information differences in the countries where amitriptyline is marketed. In 2015, the National Organization for Medicines (EOF), the Greek medicines regulator, referred this matter to the Committee for Medicinal Products for Human Use (CHMP) which on 23 February 2017 completed a review of Saroten and concluded that there is a need to harmonise the prescribing information for amitriptyline in the EU[48].

During the course of the procedure for the harmonisation of amitriptyline Summary of Product Characteristics (SmPCs), in accordance with Article 30(1) of Directive 2001/83/EC, as amended, it was of particular interest to observe the process whereby *List of Questions* are relayed between MAHs and the CHMP. As an example, a 'chronic pain' indication for amitriptyline was considered too broad. In the responses to the *List of Outstanding Issues*, the MAHs proposed the following indication: 'treatment of

[48] European Medicines Agency. Questions and answers on Saroten and associated names (amitriptyline) - Outcome of a procedure under Article 30 of Directive 2001/83/EC [Online]. EMA/118128/2017 rev1 EMEA/H/A-30/1430 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Saroten_30/WC500222211.pdf.

neuropathic pain in adults', which was the final endorsed wording[49]. For oral treatment of depression, the endorsed text was: 'treatment of major depressive disorder in adults'.

The CHMP reviewed the data submitted by MAHs and available literature in support of the proposed harmonisation, and recommended variation to the product information. Of relevance to this research are the two new sections added as warning on administration of amitriptyline to known poor metabolisers of CYP2D6 or CYP2C19 and co-administration with Cytochrome P450 inhibitors of CYP2D6. The assessment report[50] identifies the article by Hicks (Hicks et al, 2013), representing the dosing recommendations by the Clinical Pharmacogenetics Implementation Consortium (CPIC), as the basis for this opinion. The harmonised SmPC, as per the CHMP opinion, includes important pharmacogenetic considerations. Excerpts are reproduced hereunder:

"4.2 Posology and method of administration

Special populations

Cytochrome P450 inhibitors of CYP2D6
Depending on individual patient response, a lower dose of amitriptyline should be considered if a strong CYP2D6 inhibitor (e.g. bupropion, quinidine, fluoxetine, paroxetine) is added to amitriptyline treatment.

Known poor metabolisers of CYP2D6 or CYP2C19
These patients may have higher plasma concentrations of amitriptyline and its active metabolite nortriptyline. Consider a 50% reduction of the recommended starting dose.

5.2 Pharmacokinetic properties

Biotransformation

In vitro the metabolism of amitriptyline proceeds mainly by demethylation (CYP2C19, CYP3A4) and hydroxylation (CYP2D6) followed by conjugation with glucuronic acid. Other isozymes involved are CYP1A2 and CYP2C9.

Polymorphism

The metabolism is subject to genetic polymorphism (CYP2D6 and CYP2C19)."

[49] European Medicines Agency. Fourth CHMP list of outstanding issues - To be addressed by the marketing authorisation holders for Saroten and associated names; Procedure no: EMEA/H/A-30/1430. EMA/CHMP/853474/2016.
[50] European Medicines Agency. Assessment report Referral under Article 30 of Directive 2001/83/EC [Online]. Committee for Medicinal Products for Human Use (CHMP); EMA/255467/2017 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Saroten_30/WC500227970.pdf.

Additionally, in the section *Patients with special risks*, the package leaflet highlights the following: "Patients with liver diseases or people known as 'poor metabolisers' usually receive lower doses. Your doctor may take blood samples to determine the level of amitriptyline in the blood". *The Commission implementing decision of 8.5.2017 concerning, in the framework of Article 30 of Directive 2001/83/EC of the European Parliament and of the Council, the marketing authorisations for "Saroten and associated names", medicinal products for human use which contain the active substance "amitriptyline"*[51] states that Member States shall amend national marketing authorisations for Saroten and associated names on the basis of the scientific conclusions and the changes to the SmPC, the labelling and the package leaflet set out. The Decision is addressed to Member States which shall take account of the said conclusions for the assessment of the efficacy and safety of medicinal products containing amitriptyline.

The search for the term 'amitriptyline' in the medicines database of the Medicines and Healthcare Products Regulatory Agency (UK) yielded 33 different Marketing Authorisation Numbers with their corresponding Summaries of Product Characteristics. The SmPCs which had been revised to include the dosing recommendation for poor metabolisers, as per the outcome of the referral procedure, were identified by direct inspection for the phrase "*Known poor metabolisers of CYP2D6 or CYP2C19* - These patients may have higher plasma concentrations of amitriptyline and its active metabolite nortriptyline. Consider a 50% reduction of the recommended starting dose". This text appeared in 20 SmPCs, with their date of revision ranging from June 2017 to August 2018. This implies that 39% (13) of the SmPCs available on the MHRA website, were not updated with the relevant text. These SmPCs had their last revision of text in 2016

[51] European Commission. Commission Implementing Decision of 8.5.2017 [Online]. Brussels; 2017 [accessed 2019 Jul 28]. Available from: https://ec.europa.eu/health/documents/community-register/2017/20170508137542/dec_137542_en.pdf.

except for 3 SmPCs for which the text was revised in 2018 (post-referral conclusion) but still did not include the updated information. Disparities in product information is one of the demerits attributed to the scattered system tantamount to nationally authorised products – amitriptyline being a substantiating example.

3.1.2 Safety appraisals: amitriptyline interactions and adverse reaction reports

Over eight million patients worldwide – 8,158,237 – are estimated to have used amitriptyline from 1988 to 2014[52], cumulatively in marketing experience. The EU reference date (EURD) for amitriptyline is 31/07/1961, corresponding to the earliest marketing authorisation of a medicine containing amitriptyline. The European Medicines Agency maintains a list of EURDs[53], frequency for submissions of Periodic Safety Update Reports (PSURs) and related data lock points, to facilitate the single assessment of PSURs for medicinal products having the same active substances. MAHs for amitriptyline products are required to submit PSURs every three years, according to the dates published in the EURD list. Greece is the Lead Member State of the PSUR single assessment procedure for amitriptyline.

The internal documentation obtained through the Greek national competent authority and EMA official publications, related to the PSUR single assessment (PSUSA) procedure for amitriptyline, PSUSA/00000168/201501, were reviewed from the start of evaluation in May 2015 to the drafting of the Lead Member State preliminary Assessment Report, followed by consideration of MAHs comments and the updated Assessment Report, with

[52] Pharmacovigilance Risk Assessment Committee. PRAC Minutes of the meeting on 05-08 March 2018 [Online]. Inspections, Human Medicines Pharmacovigilance and Committees Division; EMA/288259/2018 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Minutes/2018/05/WC500248910.pdf.

[53] European Medicines Agency. Periodic Safety Update Reports [Online]. EMA [accessed 2018 Aug 16]. Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/document_listing/document_listing_000361.jsp&mid=WC0b01ac058066f910.

the final PRAC assessment report and recommendation being adopted in October 2015. The CMDh[54] scientific conclusions and grounds for the variation, amendments to the product information and timetable for the implementation were published in December 2015. Amendments to be included in the relevant sections of the SmPC involved a warning on 'QT interval prolongation', and 'electrocardiogram QT prolonged' as a common adverse reaction. The PSUSA is not a tool for harmonisation of product information but rather an exercise to update safety specifications if important new risks are identified.

The example of amitriptyline is not an isolated case. Based on the assessment of PSURs and PSUSAs, PRAC issued 842 recommendations in 2017, almost one fifth of which led to changes in the product information, optimising effective and safe use of medicinal products. The EudraVigilance database collects ADRs in a single portal and the data analysis tools support signal detection. In pharmacovigilance, the evaluation of safety signals is routinely undertaken to determine whether a causal relationship that warrants regulatory action exists between the reported adverse event and the medicine. The EMA reviewed 2,062 potential signals in 2017, with 82% originating from EudraVigilance database monitoring. The PRAC prioritised and assessed 82 confirmed signals, with 33 of the signals which had their review completed by end of 2017 leading to an update to the product information – package leaflet for patients and SmPC for prescribers (European Medicines Agency, 2017).

[54] CMDh. Scientific conclusions and grounds for the variation, amendments to the product information and timetable for the implementation - active substance: amitriptyline. PSUSA/00000168/201501 [Online]. EMA; 2015 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Periodic_safety_update_single_assessment/2016/10/WC500214414.pdf.

In 2017, amitriptyline featured on the PRAC agenda[55] with regards to a potential association with drug-induced liver injury or hepatocellular injury. The Pharmacovigilance Risk Assessment Committee agreed that, in their routine safety surveillance, Marketing Authorisation Holders for products containing amitriptyline should continue monitoring these events but no further regulatory action was deemed necessary in view that the product literature already included terms to encompass the risk. Following the publication by Taavola[56] in 2017, a signal of dry eye was identified by the Greek national competent authority, requiring analysis and prioritisation by the PRAC. In the April 2018 PRAC meeting[57], considering the evidence in EudraVigilance and in the literature, as well as the MAHs' comments on a proposed update with regards to the risk of dry eye associated with amitriptyline, the PRAC agreed that the MAHs of products containing amitriptyline should submit, within two months, a variation to the relevant national competent authorities, to add the undesirable effect 'dry eye' with a frequency not known, in the product information.

The evaluation conducted in this study focussed on cases of suspected adverse reactions with amitriptyline, accessed through EudraVigilance, reporting 'drug-interaction' as a reaction in the submission. A total of 440 other drugs featured in the 554 cases extracted. Table 3-1 portrays the approach adopted and an example of the causality assessment carried out for one case. Bupropion, quinidine, fluoxetine, paroxetine, thioridazine, and/or tramadol were involved in 73 of the 221 cases considered (33%). Drug interactions

[55] Pharmacovigilance Risk Assessment Committee. PRAC Minutes of the meeting on 23 - 26 October 2017 [Online]. Inspections, Human Medicines Pharmacovigilance and Committees Division; EMA/PRAC/782491/2017 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Minutes/2018/01/WC500240971.pdf.
[56] Taavola H. Amitriptyline and dry eyes – an ADR overlooked in labelling. WHO Pharmaceuticals Newsletter No.5, 2017: World Health Organization; 2017.
[57] Pharmacovigilance Risk Assessment Committee. PRAC Minutes of the meeting on 09-12 April 2018 [Online]. Inspections, Human Medicines Pharmacovigilance and Committees Division; EMA/PRAC/288660/2018 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Minutes/2018/06/WC500250540.pdf.

involving amitriptyline and one of the CYP2D6 inhibitors/substrates which, as per the amitriptyline SmPC, may result in altered metabolism, were assessed.

Table 3-1: Assessment of 'Drug Interaction' signal for amitriptyline

Drug	Amitriptyline	
Signal	Drug Interaction	
Rationale	Assessment of reported drug interactions involving amitriptyline and one of the CYP2D6 inhibitors/substrates which, as per the amitriptyline SmPC, may result in altered metabolism.	
Source	EudraVigilance database	
Number of reports	554 Cases extracted from EudraVigilance	
	221 Cases considered following exclusion of duplicates and cases with missing data (e.g. narrative, concomitant drugs)	
Overview	Cases reported per country Australia (14), Belgium (2), Brazil (2), Canada (7), China (1), Croatia (2), Cyprus (1), France (7), Germany (31), India (1), Ireland (2), Italy (5), Japan (23), Malaysia (1), Netherlands (17), New Zealand (2), Norway (2), Poland (2), Spain (4), Sweden (4), Switzerland (11), Thailand (3), United Kingdom (4), United States (73)	
	Patient Age	Range: 7-92 years, Mean 55.5, Median 58
	Patient Gender	141 (64%) Female, 80 (36%) Male
	Serious	211 (95%) Yes, 10 (5%) No
	Number of suspect/ interacting drugs	Range: 1-19, Mean 4, Median 3
Cases assessed, in line with rationale	73 cases including bupropion (6), quinidine (1), fluoxetine (13), paroxetine (17), thioridazine (2), and/or tramadol (42)	
Expectedness	Amitriptyline SmPC Section 4.5 *CYP2D6 inhibitors:* The CYP2D6 isozyme can be inhibited by a variety of drugs, e.g. neuroleptics, serotonin reuptake inhibitors, beta blockers, and antiarrhythmics. Examples of strong CYP2D6 inhibitors include bupropion, fluoxetine, paroxetine and quinidine. These drugs may produce substantial decreases in TCA metabolism and marked increases in plasma concentrations. Consider to monitor TCA plasma levels, whenever a TCA is to be co-administered with another drug known to be an inhibitor of CYP2D6. Dose adjustment of amitriptyline may be necessary. *Thioridazine*: Co-administration of amitriptyline and thioridazine (CYP2D6 substrate) should be avoided due to inhibition of thioridazine metabolism and consequently increased risk of cardiac side effects. *Tramadol*: Concomitant use of tramadol (a CYP2D6 substrate) and tricyclic antidepressants (TCAs), such as amitriptyline increases the risk for seizures and serotonin syndrome. Additionally, this combination can inhibit the metabolism of tramadol to the active metabolite and thereby increasing tramadol concentrations potentially causing opioid toxicity.	

Example: Brief description of one case and assessment carried out

EV Safety Report Identifier: *EU-EC-1782232*

7 year old male seen in the casualty department of hospital after threatening to kill himself and threatening violence to others; incidentally found to have an elevated blood pressure (136-149/72-89); very restless, agitated, flushed and tachycardic, as well as non-complaint and aggressive. Psychiatric consultation was sought.

He was found to have a turbulent social background, characterised by considerable violence and abuse; seen by numerous paediatric and child psychiatry services; admitted several months earlier to a local hospital, but had been rapidly discharged following an altercation on the ward; diagnosis of attention deficit hyperactivity disorder and conduct disorder had been made; treatment with stimulant medication was said to have failed; treatment was instituted with amitriptyline 100 mg and clonidine 150 ug daily.

At the time of admission, fluoxetine 20 mg daily had been added to this regimen; his elevated blood pressure was fully investigated during the present admission, including normal mid-stream urine, full blood count, urinary catecholamines and thyroid function tests; abdominal computed tomography and renal ultrasound were normal; a provisional diagnosis of 'serotonin syndrome' was made.

The fluoxetine was ceased and the amitriptyline was also slowly tapered and ceased; at discharge 2 weeks later, his blood pressure normalised to 110-120/40-70; the patient continued to manifest severe behavioural symptoms but these had settled somewhat, 5 weeks after the original admission; psychosocial interventions were only partially successful, but were continued.

The report concludes that, in retrospect, serum amitriptyline levels would have been useful in consolidating the diagnosis. In this case, fluoxetine may have led to increased amitriptyline levels and thus increased the serotonin level, giving rise to a serotonin syndrome.

Causality assessment

The presenting symptoms are suggestive of serotonin syndrome. Risk factors: high dose of amitriptyline (100 mg daily; SmPC recommends 10 mg – 20 mg daily for children aged 6-10); concomitant fluoxetine (CYP2D6 inhibitor; may further increase amitriptyline concentrations).
Possible: the event occurred within a reasonable time sequence to the co-administration of the drugs, but which could also be relatively explained by the underlying condition. Information on drug withdrawal is unclear to interpret, in that both amitriptyline and fluoxetine were discontinued.

Comments
The narrative of the case summarised above was submitted by a manufacturer in 1997, citing a published article which reported this case in the Journal of Paediatrics and Child Health (1996). Tracing back to the original paper by Levy F, Einfeld S, and Looi J (Australia), it is noted that, the authors had mentioned that fluoxetine inhibits CYP2D6, the enzyme responsible for metabolising amitriptyline, and that 7% of Caucasians may lack CYP2D6 due to genetic alterations.

The reports ranged from brief texts consisting of a few lines to long cases with ample details. Drug concentrations were more likely to be investigated in the post-mortem analysis of cases leading to death (15 reactions reported were fatal). Figure 3-1 illustrates the range of years during which the 73 assessed reports were submitted, stratified by the reported seriousness of each case. Practically half of the patients were hospitalised for the reported event. A total of 55 cases (75%) scored as 'Possible', implying that the clinical event occurred within a reasonable time sequence to the administration of amitriptyline and the CYP2D6 substrate/inhibitor, but which could also be explained by concurrent disease, and the information on drug(s) withdrawal was lacking or unclear. Figure 3-2 depicts the outcome of the causality assessment as per the described protocol. Assigning a 'Probable' or 'Highly Probable' score was often implausible particularly because the drugs which were suspected to be contributing to the adverse reaction were generally withdrawn at the same time, with no subsequent re-challenge.

Figure 3-1: Reports stratified by year of submission and seriousness

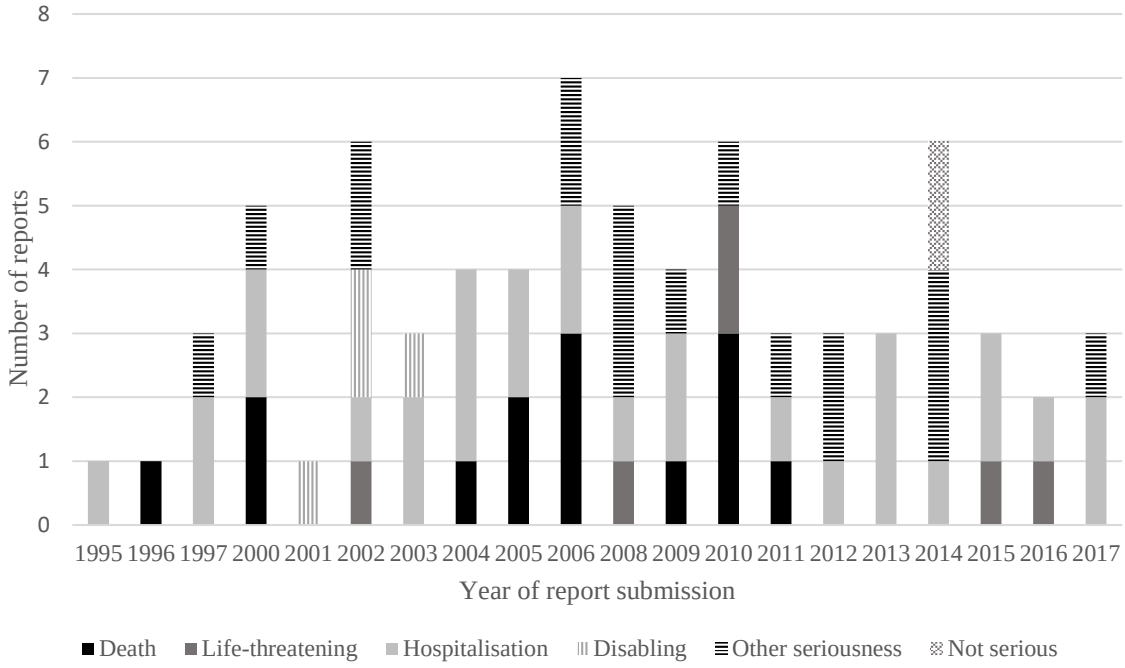
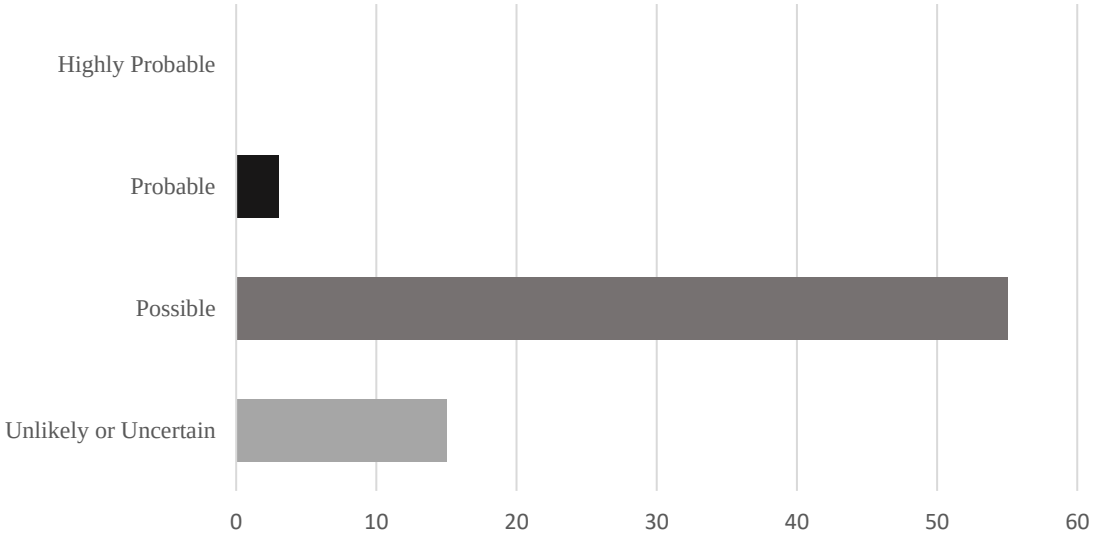


Figure 3-2: Outcome of causality assessment per number of cases



	Unlikely or Uncertain	Possible	Probable	Highly Probable
Number of cases	15	55	3	0

Cases were investigated with particular attention to drugs clearly listed in the SmPC for their potential to affect amitriptyline, or themselves being affected by amitriptyline. As observed, patients may be administered multiple medications at any one point in time, which may impact on the course of clinical outcomes. The implications of genetically determined CYP activity and the potential of genotyping was hardly mentioned in any of the cases. Out of the 73 cases assessed, 11 reports (15%) made reference to CYP enzymatic metabolism and potential inhibition, whereas only two narratives mentioned genetic testing, stating as follows, 'the patient possessed a fully functional cytochrome P450 (CYP) 2D6 enzyme' and 'genetic tests did not show any abnormal result'.

The overall outcome indicates that drug interactions at the CYP2D6 enzyme level are indeed possible, and may be associated with important reactions such as confusional state, cardiac arrest, seizures, and serotonin syndrome, as a result of, inter alia, phenoconversion, decreased metabolism and increased plasma concentrations. Thirty-five (35) of the cases included reference to additional information, from follow-up

reports, to update the case. The rate of follow-up reports was almost 50% for the cases assessed. It is important to note that reports with missing data, which are likely to also lack follow-up reports, had already been excluded, and thus the rate of not submitting follow-up reports is conceivably higher than 50%.

The fragmentary nature of submitted reports was also evident when reviewing Individual Case Study Reports (ICSRs) whereby a high proportion had no dose specified, hindering practical evaluation. A total of 391 ICSRs were retrieved from EVDAS on 6 March 2019; 310 ICSRs for amitriptyline and PT "dry mouth" and 81 ICSRs for amitriptyline and PT "sedation". A total of 164 cases of dry mouth were reported in patients on a daily amitriptyline dose of 10 mg (54, 32.9%) or 25–75 mg (110, 67.1%). A total of 28 cases of sedation were reported in patients on a daily amitriptyline dose of 10 mg (4, 14.3%) or 25–75 mg (24, 85.7%). The outcomes, as summarized in Table 3-2, point towards higher reporting rates of both dry mouth and sedation for patients receiving 25–75 mg amitriptyline daily, as compared to a 10 mg daily dose. The patient population receiving 25–75 mg daily doses is possibly larger than that of patients prescribed 10 mg daily, which may in turn affect the estimates, just as could be the case for the numerous reports with unknown dose.

Table 3-2: Individual Case Study Reports for amitriptyline and *dry mouth* or *sedation*

Dry Mouth, *N* = 310					
Daily Dose	10 mg	<10 or >10 but <25 mg	25–75 mg	>75 mg	Unknown
n	54	15	110	22	109
Sedation, *N* = 81					
Daily Dose	10 mg	<10 or >10 but <25 mg	25–75 mg	>75 mg	Unknown
n	4	3	24	15	35

3.2 Analytical developments

Analytical and technical requisites for the application of a pharmacogenetic approach to guide decisions in practice were studied. The outcomes from high performance liquid chromatography experimentation are presented at the outset, while the chromatograms attained with each mobile phase are available in the published article[58]. Performance of the newly developed and validated LC-MS/MS method for the rapid, simultaneous quantification of amitriptyline, nortriptyline and their hydroxy-metabolites in human serum, applicable to therapeutic drug monitoring, is described in Section 3.2.2. Buccal swabs rendered effective sources for extracting DNA and TaqMan® genotyping of *CYP2D6* and *CYP2C19*. The genotype and metabolizer status inferred by laboratory reports are thereafter considered in line with the 2019 CPIC consensus of CYP2D6 genotype to phenotype, enabling pragmatic construal of standardisation concerns.

3.2.1 High performance liquid chromatography

An Agilent 1260 Infinity Series® II liquid chromatography system, at the Department of Pharmacy of the University of Malta, was employed in the development of an HPLC method for the separation of similar tricyclic compounds – amitriptyline, nortriptyline and the hydroxy-metabolites. Parameters were systematically assessed, experimenting with column temperatures, different injection volumes and flow rates.

A systematic technique for the simultaneous chromatographic separation of amitriptyline and nortriptyline was first developed, scrutinising the combined effect of two major analytical parameters, buffer pH and mobile phase composition. At pH 4.4,

[58] Mifsud Buhagiar L, et al. Implications of mobile phase composition and pH on the chromatographic separation of amitriptyline and its metabolite nortriptyline. *Int J Pharm Pharm Sci* 2018;10(4):132-8. Available from: https://innovareacademics.in/journals/index.php/ijpps/article/view/24817.

chromatograms showed limited separation and poor resolution, particularly with increasing acetonitrile concentrations. Significant improvement was observed at pH 5.6 while a pH of 6.8 prolonged the time for separation and resulted in undesirable peak shape. An increase in the percentage of acetonitrile decreased the retention of amitriptyline and nortriptyline. This may be explained by competitive interaction of acetonitrile with the stationary phase, diminishing the interaction of amitriptyline and nortriptyline with the stationary phase. Reducing the amount of acetonitrile delayed the elution of amitriptyline, which was most evident at pH 6.8. Analysis at pH 4.4 highlighted how increasing volumes of acetonitrile may result in decreased resolution between the two peaks.

With all the different mobile phases used, nortriptyline eluted before amitriptyline. More symmetrical peaks and less peak tailing were attained when using the mobile phase containing 35% acetonitrile at pH 5.6, with a reasonable retention time for the separation of nortriptyline and amitriptyline (eluting at 4.66 min and 5.92 min respectively). A composition of 40% acetonitrile at pH 5.6, allowed for complete separation of analytes within 4 minutes with comparable resolution. Figure 3-3 shows how retention time decreased with increasing percentage of acetonitrile when using the mobile phase at pH 5.6.

Since the pH of the mobile phase with improved performance is 3.8 pH units lower than the pKa of amitriptyline, the molecules are expected to be ionised [$R\text{-}NH^{+}(CH_3)_2$] in the buffer used, occurring predominantly in the protonated form (Figure 3-4). Nortriptyline is similar in structure to amitriptyline and has a slightly higher pKa of 9.7. This similarity intensifies the challenge of determining isocratic conditions for the separation.

Figure 3-3: HPLC – Plots of the retention time vs mobile phase acetonitrile percentage

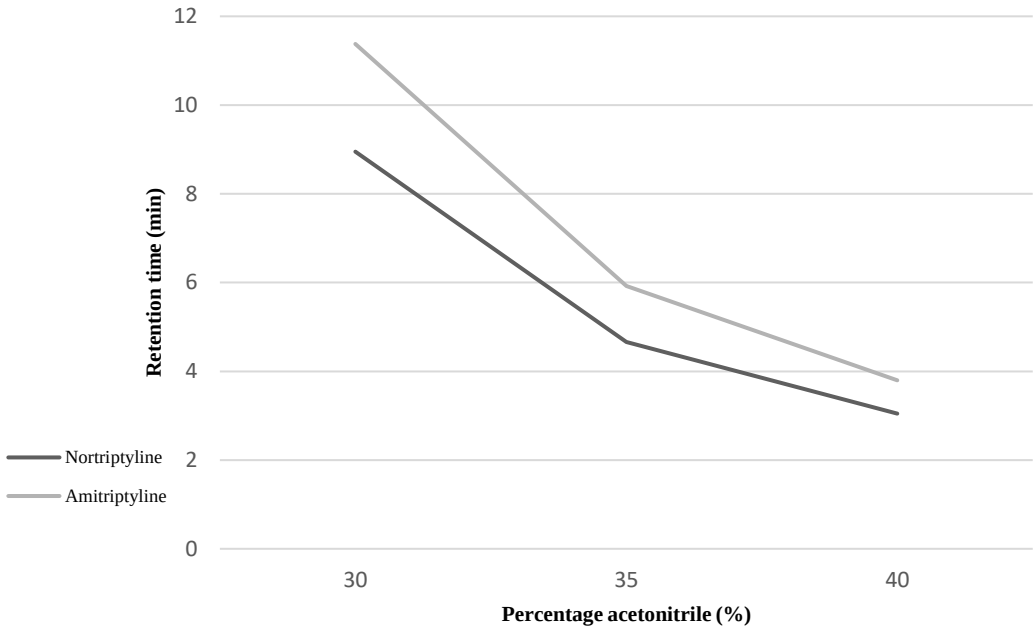
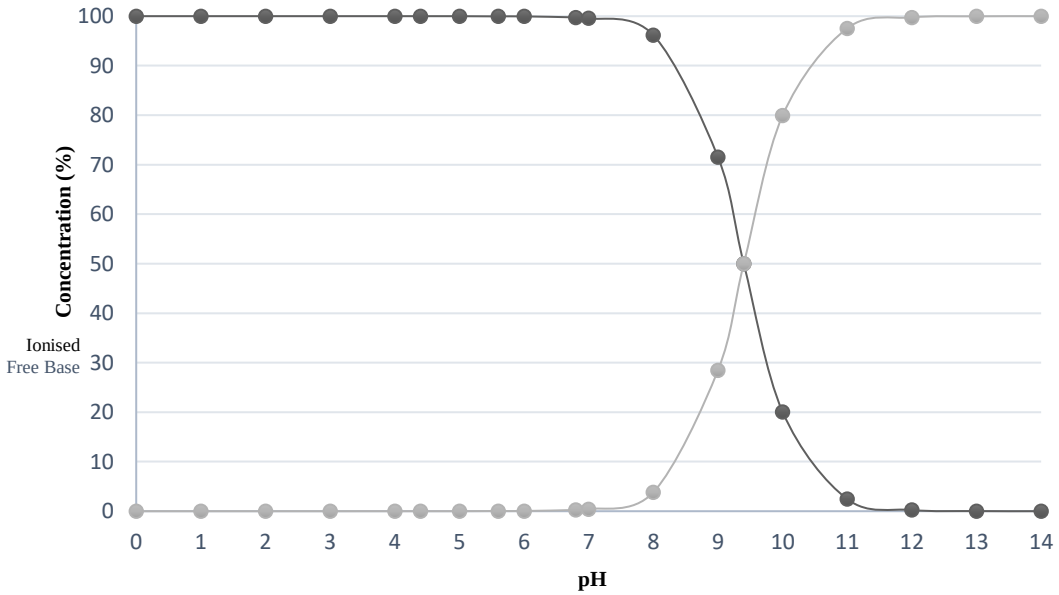
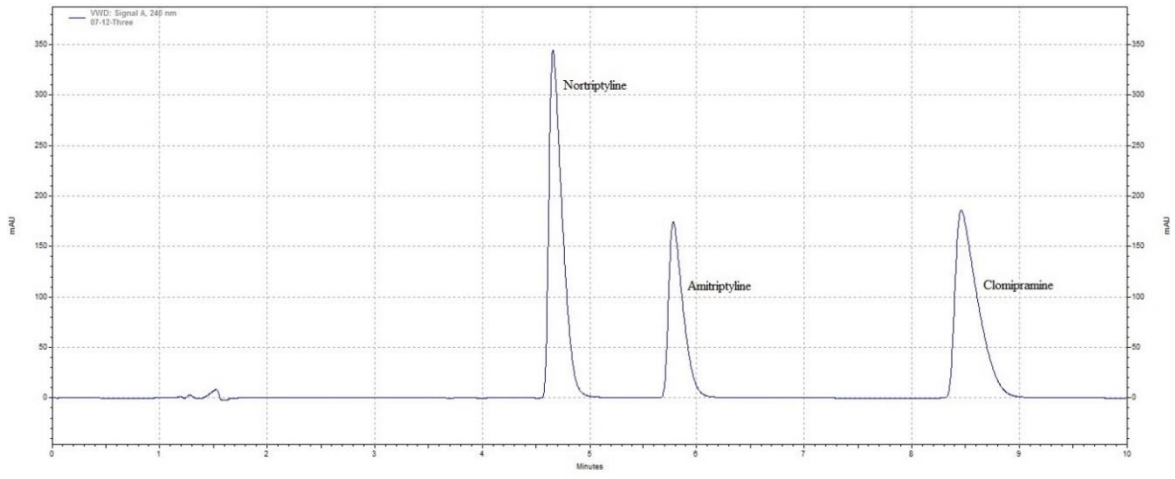


Figure 3-4: HPLC – Speciation plot for amitriptyline



In analytical reversed phase HPLC studies of amitriptyline and its metabolite nortriptyline, clomipramine characterizes a potential internal standard due to its chemical and physical similarity to the analytes of interest. Adding a known amount of clomipramine serves as a normalizing factor to compensate for losses and variability throughout the process. It is desirable that the internal standard elutes near to, but is well resolved from the calibrated compounds and is chromatographically distinguishable. The mobile phase composed of phosphate buffer and acetonitrile in the ratio of 65:35 (v/v) at pH 5.6 yielded the best compromise for the separation of amitriptyline, nortriptyline and clomipramine, as the analyte peaks were well defined and resolved at this composition. The chromatographic conditions described entail relatively low consumption of organic solvent and energy by operating around room temperature, supporting the progress towards green analytical chemistry to minimize the environmental impact. The chromatogram in Figure 3-5 shows separation detected at a UV wavelength of 240 nm. Both amitriptyline and nortriptyline had a lower intensity of absorbance at this wavelength, compared to 210 nm.

Figure 3-5: HPLC – Separation of amitriptyline, nortriptyline and clomipramine
Mobile phase: 35% acetonitrile; pH 5.6



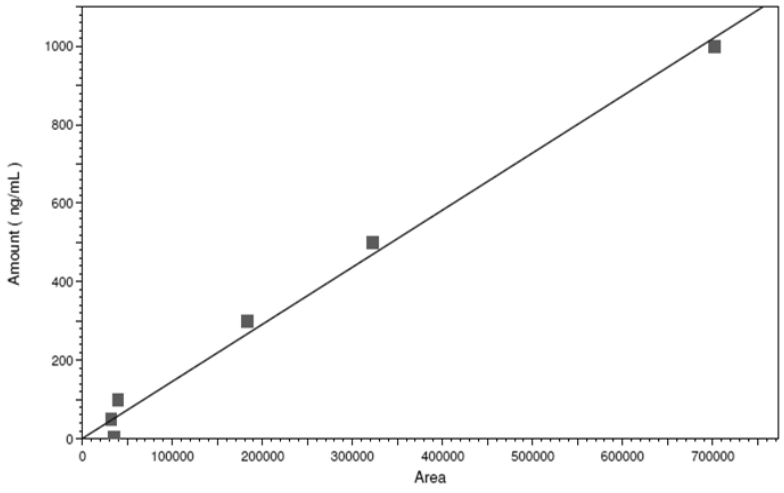
Most runs were subsequently performed using dual wavelength – 210 and 240 nm, with UV/visible absorbance providing limited selectivity as absorbance of aromatic residues is likely in this range of wavelengths. UV absorbance in the region of 210 nm and lower is not specific since most compounds holding hetero-atoms and multiple bonds absorb UV below 200-210nm (Chang & El-Shourbagy, 2009). The process undertaken shows how separation of TCAs can be optimised by concurrent modification of the amount of organic modifier and pH of the buffer, the critical parameters in reversed-phase chromatography (Bergés et al, 2000, Espinosa et al, 2002, Galaon & David, 2012). Calibration curves were constructed for amitriptyline and nortriptyline in the concentration range of 5 – 1000 ng/mL. A linear relationship was observed (Figure 3-6) between the area under the detector signal peaks and the concentrations of amitriptyline and nortriptyline.

The method was developed further for the simultaneous separation of the tricyclic hydroxy-metabolites which are often excluded from therapeutic drug monitoring even though these are associated with cardiotoxicity (Hicks et al, Suppl 2017). By attuning the critical parameters, adequate separation of these compounds, which are similar in structure, was attained (Figure 3-7). Optimal chromatographic outcomes were achieved with isocratic conditions comprising of 31% acetonitrile and 69% phosphate buffer at pH 5.6 as mobile phase, a flow rate of 0.5 mL/min and detection wavelength set at 210 nm. These parameters resulted in the separation of trans-10-hydroxynortriptyline, trans-10-hydroxyamitriptyline, cis-10-hydroxynortriptyline, cis-10-hydroxyamitriptyline, nortriptyline and amitriptyline, eluting at 4.3, 4.7, 5.4, 6.0, 15.9 and 19.9 min, respectively.

Figure 3-6: HPLC – Calibration curves for amitriptyline and nortriptyline

Calibration curve for amitriptyline; 5 – 1000 ng/mL; λ 240 nm

Average RF	RF StDev	RF %RSD	R^2	Linear Fit
0.0015	0.0008	51.9941	0.9909	ax + b a = 0.0015 b = 0.0000



Calibration curve for nortriptyline; 5 – 1000 ng/mL; λ 240 nm

Average RF	RF StDev	RF %RSD	R^2	Linear Fit
0.0284	0.2266	80.0837	0.9559	ax + b a = 0.0152 b = 0.0000

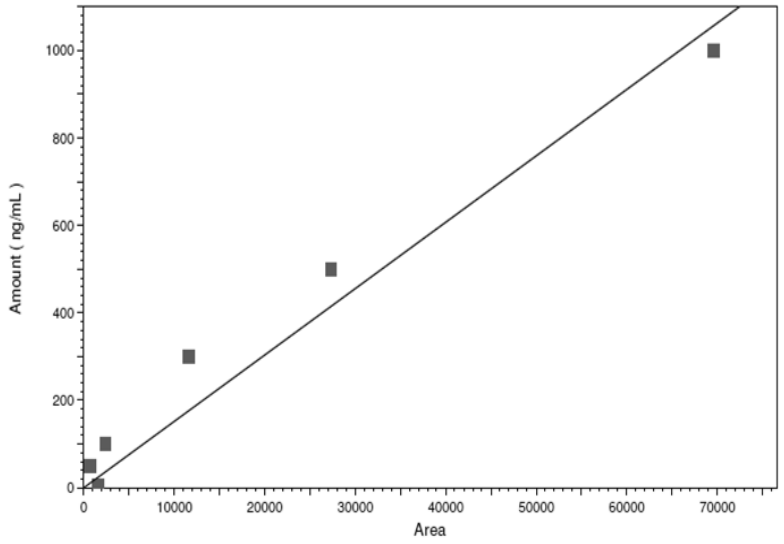
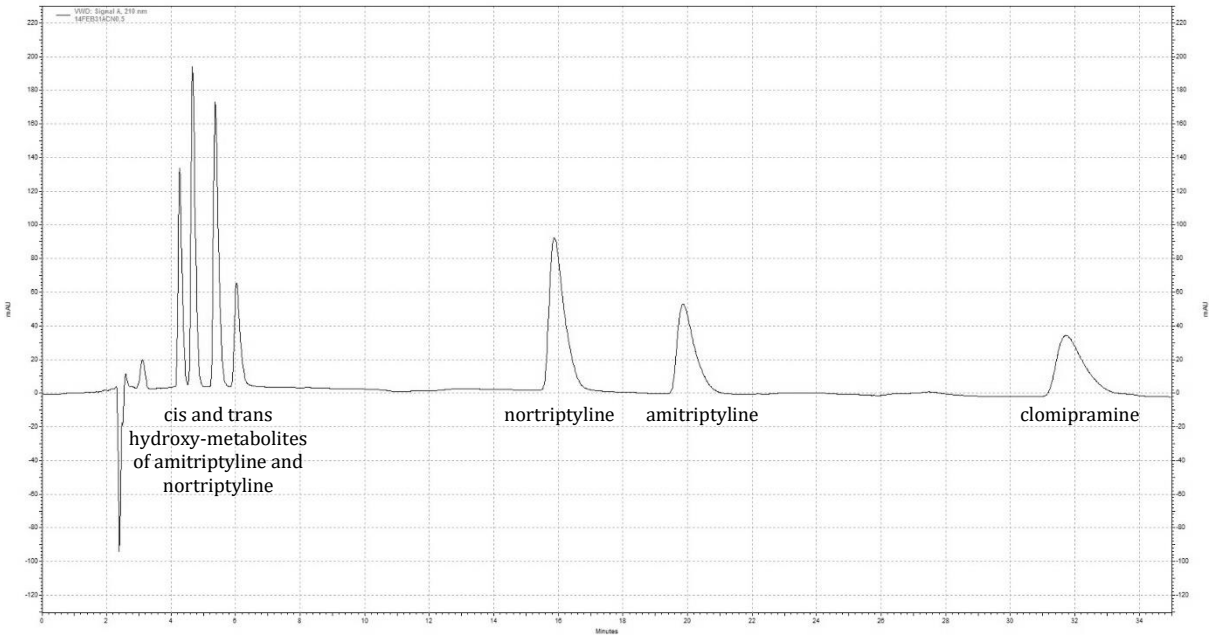


Figure 3-7: HPLC – Chromatogram showing separation of amitriptyline, nortriptyline and their hydroxy-metabolites, with clomipramine as internal standard



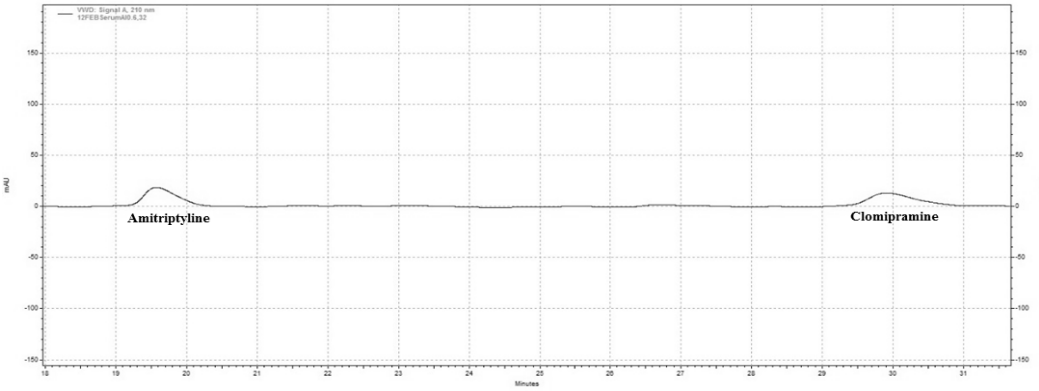
The chromatogram in Figure 3-7 resulted from 100 µg/mL solutions of standards injected in the HPLC system. The diminished absorbance observed when injecting solutions with a concentration of 1 µg/mL, moving closer to values reported in patients, makes interpretation more challenging. The method developed for simultaneous assay of the tricylic compounds is apposite for validation in the analysis of pharmaceutical impurities. The prospective applicability of the proposed procedure to pharmacokinetic studies, which are relevant when metabolite-to-parent drug concentration ratios are linked to potential variations in enzyme capacity and clinical events, was investigated further, with due consideration to the pertinent sample preparation procedures.

The analytical design of the present study is complicated by the low range of concentrations being explored in the serum matrix, in line with the estimated blood levels that one would expect in human subjects on amitriptyline therapy. Sample preparation through the liquid-liquid extraction with back extraction procedure, which involves a

second extraction step, should enable removal of unwanted matrix components, solvent exchange and analyte enrichment. Protein precipitation, in comparison, features simplicity of the method as its major advantage (Hansen et al, 2012).

The chromatogram in Figure 3-8 shows amitriptyline and clomipramine (100 µg/mL) extracted from serum, with no buffer added during protein precipitation. The chromatographic conditions included an injection volume of 10 µL, a flow rate of 0.6 mL/min and a mobile phase composed of 32% ACN and 68% phosphate buffer at pH 5.6.

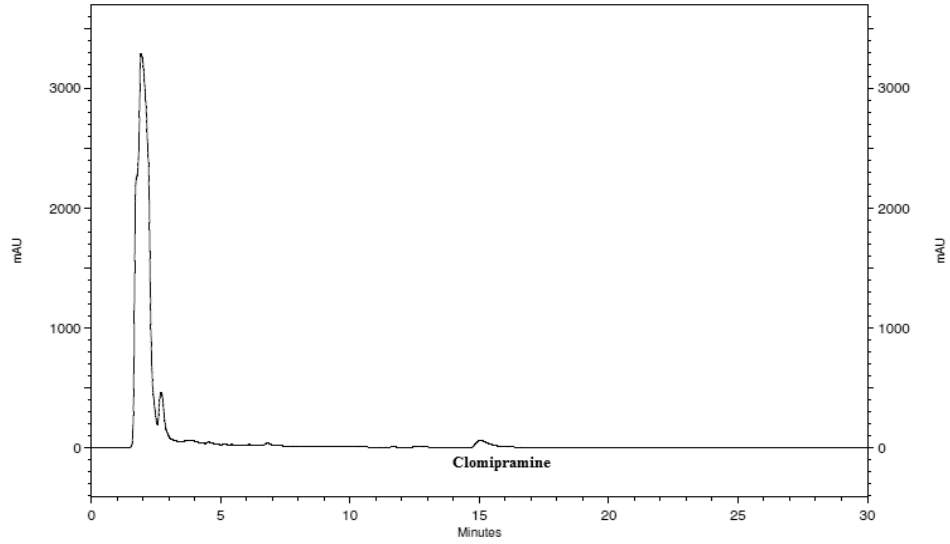
Figure 3-8: HPLC – Chromatogram for serum spiked with 100 µg/mL amitriptyline and clomipramine



Adding a drop of phosphate buffer (pH 10) appears to improve extraction of clomipramine, compared to no buffer added or the addition NaOH or a buffer with pH 5.6 or pH 9. The run depicted by Figure 3-9, completed with a flow-rate gradient, shows enhanced UV absorption for clomipramine when a drop of pH 10 buffer was added to the spiked serum at protein precipitation. Amitriptyline, present at a lower concentration of amitriptyline (1 µg/mL), proves difficult to characterise.

Figure 3-9: HPLC – Chromatogram for serum spiked with 1 µg/mL amitriptyline and 100 µg/mL clomipramine

With the addition of a drop of phosphate buffer (pH 10) during sample preparation

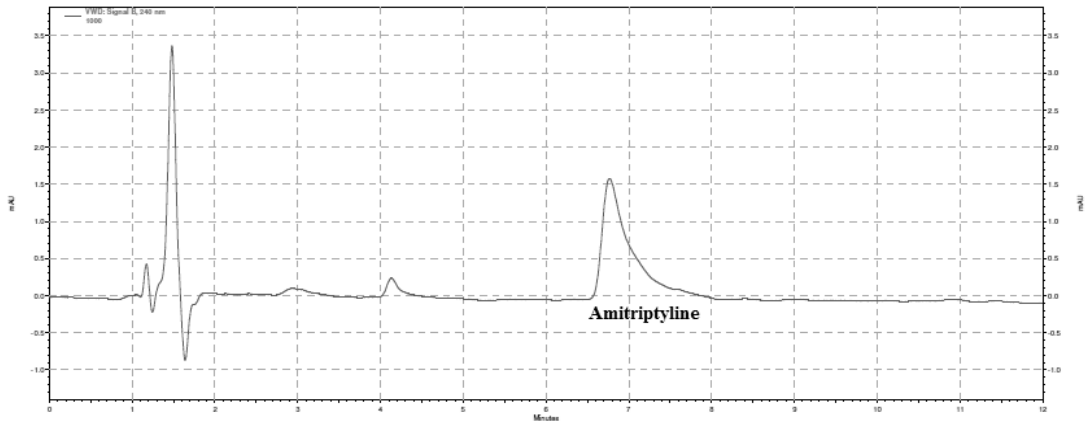


Despite attempts to reconstitute dried residues in a lower of volume of mobile phase and increasing injection volumes, outcomes from serum analysis in the range of concentrations under investigation, were quite uninterpretable. Figure 3-10 shows the chromatogram for amitriptyline standard solution at a concentration of 1000 ng/mL compared to the chromatogram of serum spiked with the same concentration of amitriptyline. Runs for samples obtained following liquid-liquid extraction and back extraction did not produce any well-defined chromatographic peaks at all different concentration levels considered.

The developed method was adapted to an Ultra-High Performance Liquid Chromatography (UHPLC) system at Universal Limited, Malta Life Sciences Park, to investigate potential improvement in peak resolution and quantification of the lower concentrations. Detection at 210 and 240 nm showed identifiable peaks for the 100 µg/mL solution containing the hydroxy-metabolites, amitriptyline, nortriptyline and clomipramine (Figure 3-11). Response at lower concentrations was still minimal.

Figure 3-10: HPLC – Comparison between chromatogram for amitriptyline standard solution and chromatogram for serum spiked with amitriptyline standard solution

Chromatogram for standard solution of amitriptyline at a concentration of 1000 ng/mL



Chromatogram for serum spiked with amitriptyline at a concentration of 1000 ng/mL

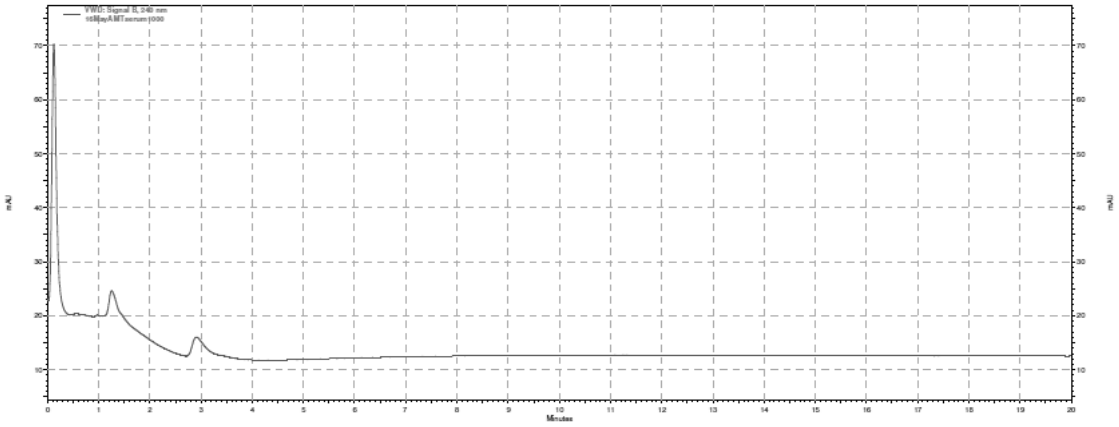
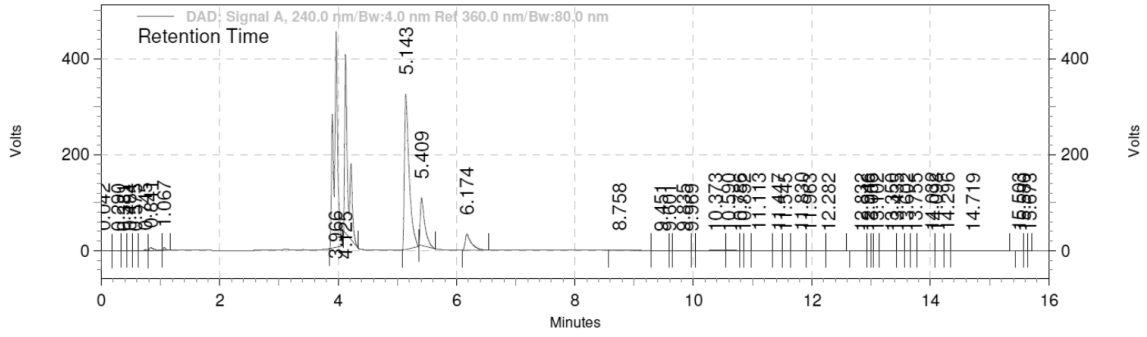


Figure 3-11: UHPLC – Chromatogram using Ultra-High Performance Liquid Chromatography system



3.2.2 Liquid chromatography tandem mass spectrometry

LC-MS/MS method development ensued at the Toxicology Laboratory of Mater Dei Hospital. Table 3-3 gives an overview of the retention times, MRM transitions and optimized parameters for the analytes under study and internal standards. The presence of isobaric metabolites necessitated physical separation on the column.

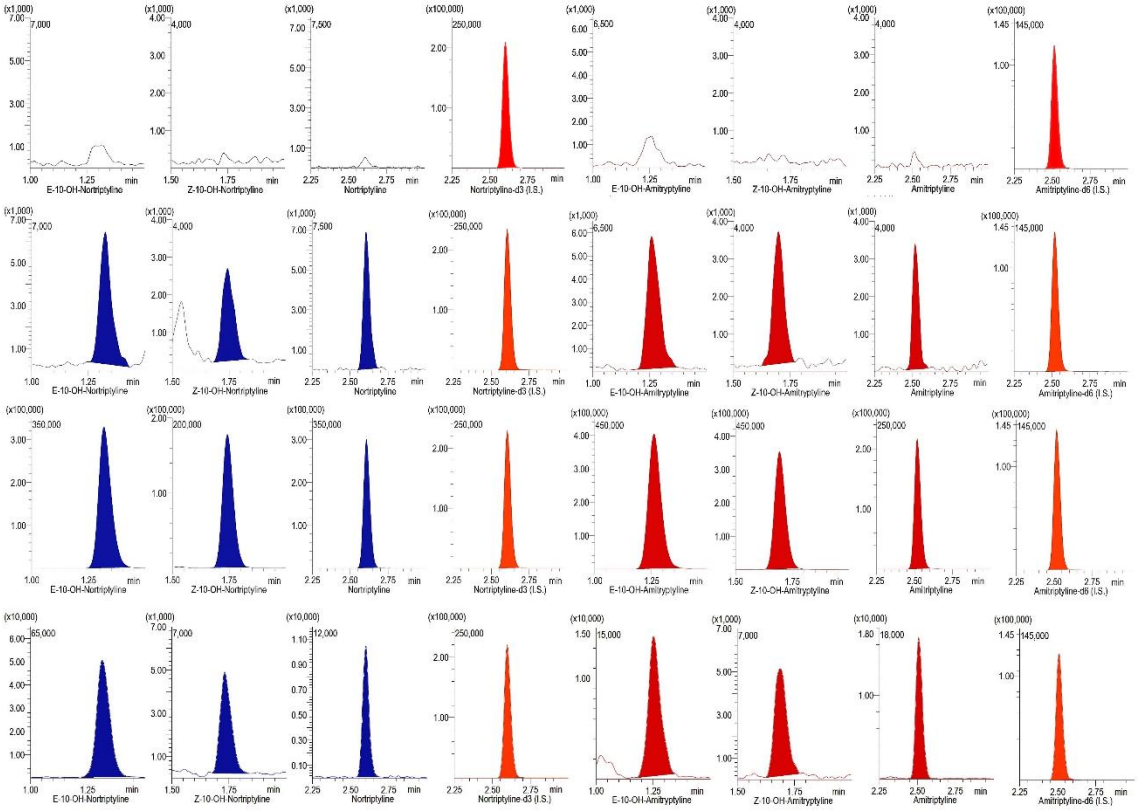
Table 3-3: LC-MS/MS – Retention times, Multiple Reaction Monitoring (MRM) transitions and optimized parameters

Analyte	**LC-**	**MS/MS conditions**							
		Precursor ion		Product quantitation ions			Product reference ions		
	Retention time (min)	*m/z*	Q1 pre-bias (V)	*m/z*	CE (V)	Q3 pre-bias (V)	*m/z*	CE (V)	Q3 pre-bias (V)
Amitriptyline	2.50	278.00	-20	233.15	-16	-29	117.15	-22	-24
Nortriptyline	2.59	264.00	-30	233.00	-13	-18	117.20	-19	-25
Z-OH NOR	1.71	280.00	-14	262.20	-12	-20	215.00	-40	-24
E-OH NOR	1.28	280.00	-20	262.10	-11	-20	215.00	-40	-25
Z-OH AMI	1.66	294.00	-21	276.10	-14	-21	215.00	-44	-25
E-OH AMI	1.21	294.00	-23	276.30	-15	-16	215.15	-47	-26
Amitriptyline-D6	2.50	284.10	-14	233.15	-17	-18	117.05	-30	-25
Nortriptyline-D3	2.59	267.00	-30	233.20	-14	-18	105.05	-19	-13

Run time, including equilibration, was of 6 minutes per sample for the simultaneous quantification of amitriptyline, nortriptyline and their hydroxy-metabolites. Sample preparation was a simple protein precipitation step. The use of cooled acetonitrile (-20°C) was preferred to room temperature acetonitrile as a more compact insoluble pellet was produced with the added advantage of minimal temperature increase during the centrifugation stage. Figure 3-12 portrays outcomes, achieved at the identified optimal working conditions, for the blank, LLOQ concentration, 50 ng/mL standard, and serum sample withdrawn from patient on amitriptyline therapy.

Figure 3-12: LC-MS/MS – Filtered Multiple Reaction Monitoring (MRM) ion chromatograms

E-OH NOR (m/z 280.00>262.10), Z-OH NOR (m/z 280.00>262.20), NOR (m/z 264.00>233.00), Nortriptyline-d3 (IS, m/z 267.00>233.20), E-OH AMI (m/z 294.00>276.30), Z-OH AMI (m/z 294.00>276.10), AMI (m/z 278.00>233.15) and Amitriptyline-d6 (IS, m/z 284.10>233.15) in vertical order: sample blank (IS only), calibration standard at LLOQ concentrations, calibration standard at 50 ng/mL, and sample.



The acceptance criteria for precision and accuracy were met with CV% values of <15% for all analytes at the concentrations tested. A relatively lower nortriptyline concentration (0.2 ng/mL) showed significant bias (24%) and was rejected. Lower limit of detection (LOD) was estimated as 20 pg/mL for amitriptyline and nortriptyline and 0.1 ng/mL for the hydroxylated metabolites, from noise values. Levels ranging between 2 ng/mL and 400 ng/mL displayed comparable recovery values, with a degree of discrepancy being observed in the recovery of analytes at the LLOQ, which was considered acceptable since consistent from run to run. Table 3-4 presents linearity and recovery data. Linearity was >0.999 for all analytes in all runs and the LLOQ was identified from these results. Precision and bias data is reported in Table 3-5, while Figure 3-13 portrays typical calibration curves.

Table 3-4: LC-MS/MS – Linearity and recovery

Compound (n=6)	**IS**	**Linearity** *0.5 – 400 ng/mL* Mean r^2	**% Recovery (CV%)** LLOQ *0.5 ng/mL*	ULOQ *400 ng/mL*
Amitriptyline	Amitriptyline-D6	0.9998 ± 0.0001	61.1 (6.7)	83.2 (4.6)
Nortriptyline	Nortriptyline-D3	0.9998 ± 0.0002	96.6 (5.9)	83.4 (3.7)
Z-OH NOR	Nortriptyline-D3	0.9990 ± 0.001	93.4 (4.7)	80.3 (4.9)
E-OH NOR	Nortriptyline-D3	0.9992 ± 0.001	90.0 (7.1)	78.3 (5.0)
Z-OH AMI	Amitriptyline-D6	0.9994 ± 0.001	60.2 (9.5)	81.9 (4.2)
E-OH AMI	Amitriptyline-D6	0.9992 ± 0.001	121.5 (7.4)	80.6 (4.5)

Table 3-5: LC-MS/MS – Intra- and inter-day precision and bias

Compound (n=6)	**IS**	**Intra-day precision CV% (% bias)** LLOQ 0.5 ng/mL	50 ng/mL	ULOQ 400 ng/mL	**Inter-day precision CV% (% bias)** LLOQ 0.5 ng/mL	50 ng/mL	ULOQ 400 ng/mL
Amitriptyline	Amitriptyline-D6	3.6 (-10.2)	2.6 (0.6)	1.9 (-0.5)	3.7 (-0.6)	3.9 (1.8)	1.1 (2.1)
Nortriptyline	Nortriptyline-D3	7.0 (-3.2)	2.9 (-3.7)	1.3 (-2.2)	4.1 (-4.6)	4.4 (1.2)	1.8 (-0.9)
Z-OH NOR	Nortriptyline-D3	7.6 (15.0)	10.3 (-2.6)	1.9 (-0.8)	8.4 (-3.4)	2.6 (3.2)	2.2 (-1.4)
E-OH NOR	Nortriptyline-D3	6.2 (7.5)	13.5 (-4.1)	1.8 (-3.9)	10.2 (0.3)	3.4 (0.6)	3.8 (-2.4)
Z-OH AMI	Amitriptyline-D6	10.8 (7.4)	11.2 (-3.0)	2.6 (-0.8)	8.0 (3)	4.3 (0.3)	3.9 (0.7)
E-OH AMI	Amitriptyline-D6	6.0 (10.4)	13.9 (-2.9)	2.2 (-1.8)	2.3 (1.3)	4.3 (1.1)	5.3 (0.3)

Matrix interference was studied in six random serum samples. One of the samples contained a high bilirubin content (>170 µmol/L) whilst other samples were found to contain drugs including quetiapine and the 7-hydroxy metabolite, mianserin, venlafaxine, fluoxetine, melitracen, diazepam and nordiazepam, valproic acid, clomipramine and desmethylclomipramine, clozapine and N-oxide metabolite, and olanzapine. Nonetheless, no significant interferences were observed in the blanks and matrix effects were minimal (Table 3-6).

Figure 3-13: LC-MS/MS – Calibration curves

AMI, NOR, E-OH AMI, E-OH NOR, Z-OH AMI and Z-OH NOR for the range 0.5 – 400 ng/mL, using linear response function with 1/C weighing.

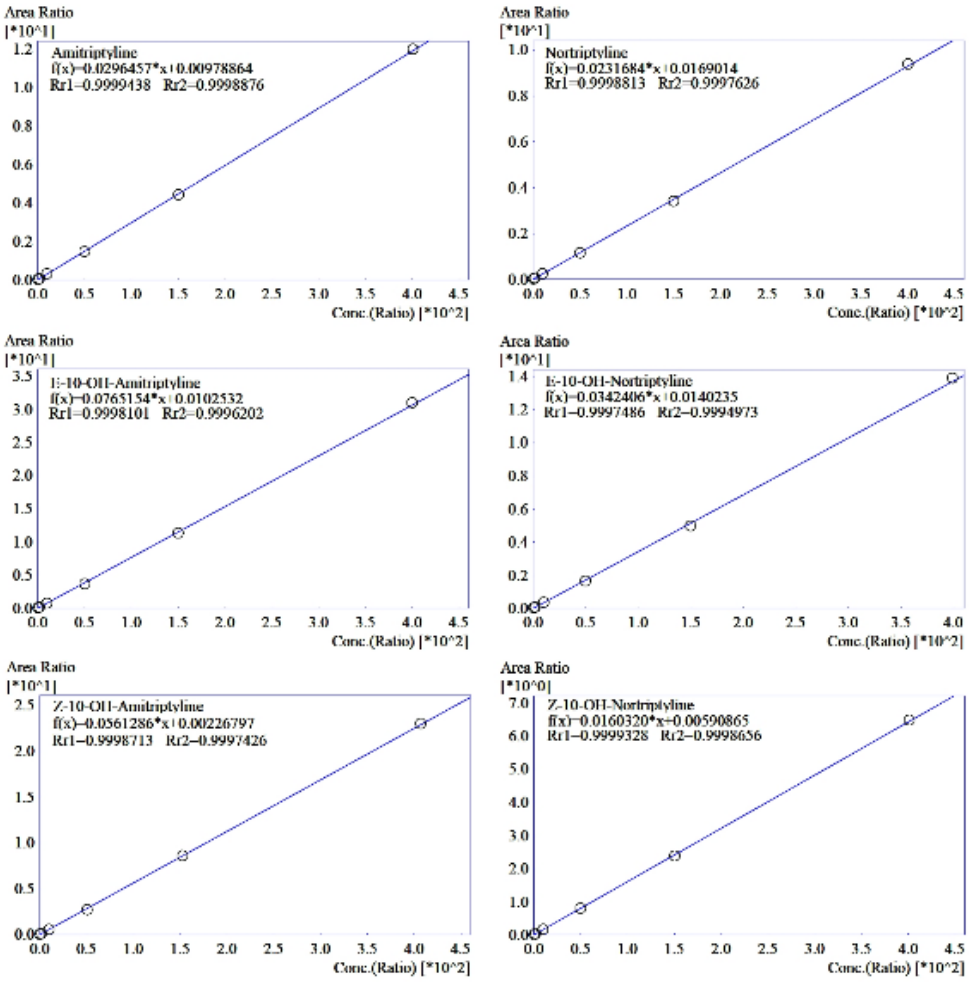


Table 3-6: LC-MS/MS – Matrix effects at the limits of quantification

Near LLOQ and ULOQ (n=6)

Compound	**IS normalised matrix factor (CV%)** 1ng/ml	350ng/ml	**IS normalised matrix factor (CV%)** 1ng/ml	350ng/ml
Amitriptyline	0.97 (8.3)	0.88 (9.2)	1.09 (3.5)	0.98 (2.1)
Nortriptyline	0.80 (13.6)	0.82 (11.0)	1.00 (6.1)	0.97 (2.2)
Z-OH NOR	0.85 (9.2)	0.88 (7.8)	1.06 (2.8)	1.05 (3.2)
E-OH NOR	0.83 (5.7)	0.88 (3.4)	1.05 (13.9)	1.05 (10.9)
Z-OH AMI	0.93 (6.4)	0.94 (4.5)	1.05 (7.5)	1.05 (5.2)
E-OH AMI	0.94 (2.2)	0.96 (1.5)	1.07 (8.8)	1.08 (7.6)
Amitriptyline-D6	0.89 (8.9)	0.90 (9.0)		
Nortriptyline-D3	0.80 (11.3)	0.84 (10.0)		

No significant carry-over (<LLOQ) was observed and any minor peaks appearing in the time windows of the analytes were rejected by the software on the basis of ion ratios differences from the set ratio. Incidentally, a highly concentrated sample (>2500 ng/mL all analytes) injected during development resulted in no carry-over being observed in the analysis of a subsequent blank.

Standard solutions, samples, calibration standards and spiked controls were aliquoted into microvials during the collection phase and stored at -30°C. No sample, standard or control were used more than once after thawing and the effects of freeze-thaw cycles were not investigated, though previous studies (Kishore Kumar et al, 2010) have shown no significant deterioration after three freeze-thaw cycles. In stability studies, the predicted concentrations for AMI, NOR and their hydroxy-metabolites in spiked serum stored at -20°C and -30°C were within the assay variability limits. The samples were analysed over a period of 8 weeks and represented concentrations at and between LLOQ and ULOQ. As expected (Bonke, & Jensen, 2010), no analyte degradation was observed during the duration of the study.

The developed method entails a simple extraction procedure, combined with a rapid and sensitive LC-MS/MS analysis. The validated method has been applied on real samples, demonstrating practical applicability in the field of clinical analysis. Table 3-7 presents the concentrations of amitriptyline and nortriptyline, in ng/mL, measured by the LC-MS/MS method developed and validated in-house, compared to the concentrations reported by the out-sourced laboratory, for aliquots from same serum sample of 44 patients. The method of analysis of the out-sourced lab had a lower limit of 10 ng/mL for both amitriptyline and nortriptyline, with the detection limits increasing to 13 ng/mL when performing analysis with reduced sample volume.

Table 3-7: Amitriptyline and nortriptyline serum concentrations, as determined by the method developed in-house and by the out-sourced lab

Sample	Amitriptyline (ng/mL)		Nortriptyline (ng/mL)	
	In-house method	Out-sourced lab	In-house method	Out-sourced lab
1	136.12	101	101.50	73
2	18.21	15	5.56	<13
3	15.27	13	23.85	20
4	99.78	70	80.55	56
5	31.07	26	22.38	19
6	79.92	65	57.48	46
7	17.57	15	3.79	<13
8	83.67	66	56.26	43
9	255.82	240	133.46	116
10	59.60	53	12.13	<13
11	6.67	<13	13.32	<13
12	7.82	<10	6.19	<10
13	15.04	<13	5.76	<13
14	28.83	22	17.01	13
15	43.86	35	10.31	<13
16	48.94	42	16.83	14
17	41.92	35	5.54	<13
18	103.79	Insufficient sample	159.78	Insufficient sample
19	32.80	33	10.46	10
20	51.10	44	83.18	65
21	20.90	15	17.88	13
22	258.04	239	322.06	264
23	165.35	146	151.03	121
24	153.82	131	25.67	20
25	21.56	18	6.99	<13
26	4.4	<10	1.31	<10
26	7.35	<10	2.71	<10
27	4.43	<10	0.95	<10
28	13.38	10	3.86	<10
29	7.74	<10	6.43	<10
30	8.12	<10	3.14	<10
31	21.9	20	4.68	<10
32	4.93	<13	0.37	<13
33	2.61	<13	1.5	<13
34	2.22	<13	1.54	<13
35	5.01	<13	2.35	<13
36	10.8	<10	5.74	<10
37	6.3	<13	5.18	<13
38	9.73	<13	7.37	<13
39	5.25	<13	1.71	<13
40	260.19	216	237.22	187
41	14.78	12	1.2	<10
42	10.37	<10	1.89	<10
43	11.16	<13	2.94	<13
44	136.12	101	101.50	73

The distribution of concentrations was skewed (Figure 3-14). Inconclusive results reported by the outsourced lab were excluded for assessing the correlation between concentrations measured by the two methods of analysis. In view of non-normality (Table 3-8), the non-parametric Spearman correlation test was applied (Table 3-9). The Spearman correlation coefficients (0.996 and 0.990 for amitriptyline and nortriptyline, respectively) are close to 1, indicating a significant positive relationship (P=0.000) between the concentrations measured by the in-house LC-MS/MS method and the concentrations reported by the out-sourced laboratory.

Figure 3-14: Distribution of amitriptyline and nortriptyline concentrations for which both in-house and out-sourced results were made available

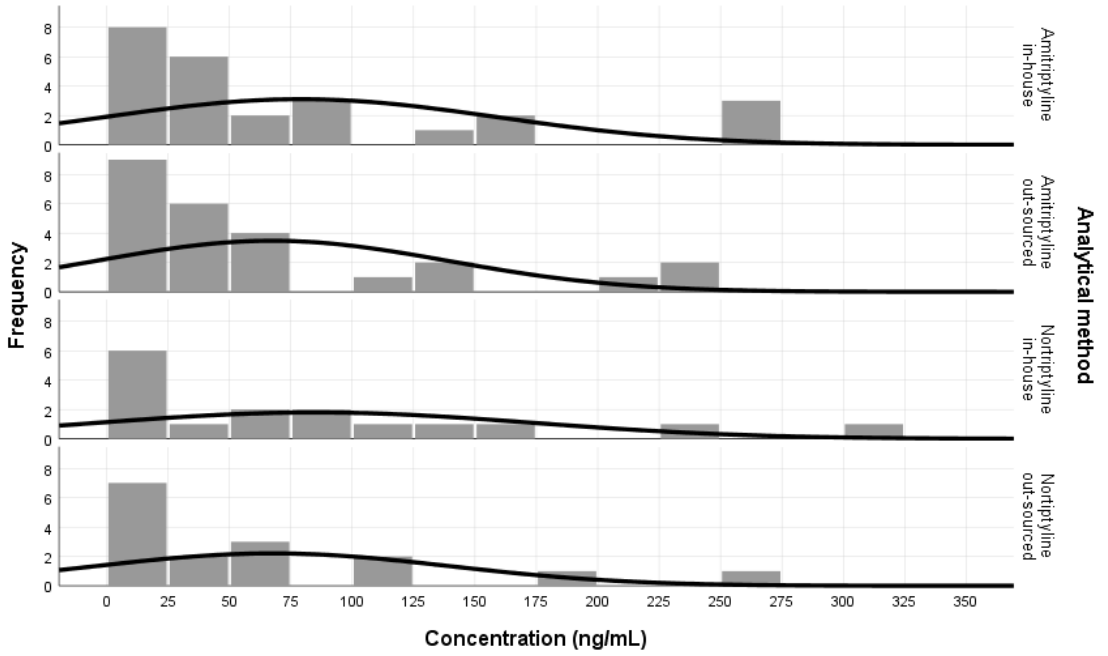


Table 3-8: Test of normality for measured concentrations

Test of Normality	Shapiro-Wilk		
	Statistic	df	P-value
Amitriptyline concentration determined by in-house LC-MS/MS method	0.853	16	0.015
Amitriptyline concentration reported by out-sourced lab	0.839	16	0.009
Nortriptyline concentration determined by in-house LC-MS/MS method	0.795	16	0.002
Nortriptyline concentration reported by out-sourced lab	0.780	16	0.002

Table 3-9: Test of correlation between amitriptyline and nortriptyline concentrations determined by in-house method and concentrations reported by the out-sourced lab

Spearman Correlation		Amitriptyline concentration (out-sourced lab)
Amitriptyline concentration (in-house)	Correlation Coefficient	0.996
	P-value	0.000
		Nortriptyline concentration (out-sourced lab)
Nortriptyline concentration (in-house)	Correlation Coefficient	0.990
	P-value	0.000

The mean (±SD) amitriptyline concentration measured by the LC-MS/MS method developed in-house was 78.98 (±80.22), while that reported by the out-sourced lab was 67.28 (±71.70). The mean (±SD) nortriptyline concentration measured by the LC-MS/MS method developed in-house was 84.80 (±88.87), while that reported by the outsourced lab was 67.50 (±72.26). The difference is statistically significant (Wilcoxon Signed Ranks Test, P=0.000), with the levels of both amitriptyline and nortriptyline being higher when measured in-house (Figures 3-15, 3-16).

Figure 3-15: Amitriptyline concentrations measured in-house vs reported by out-sourced lab

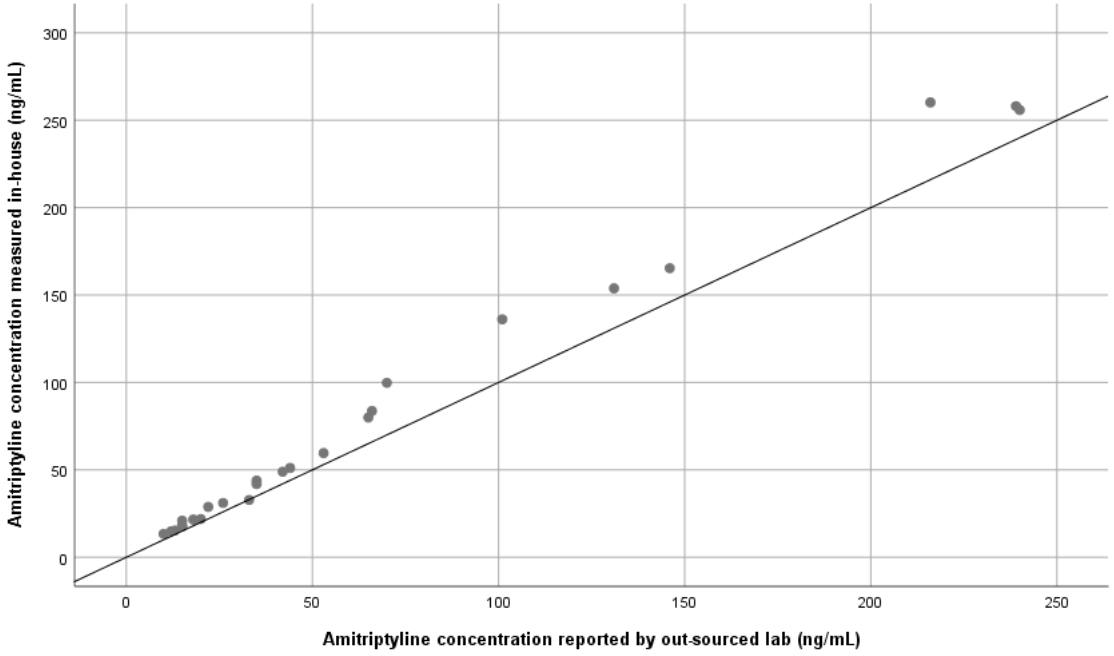
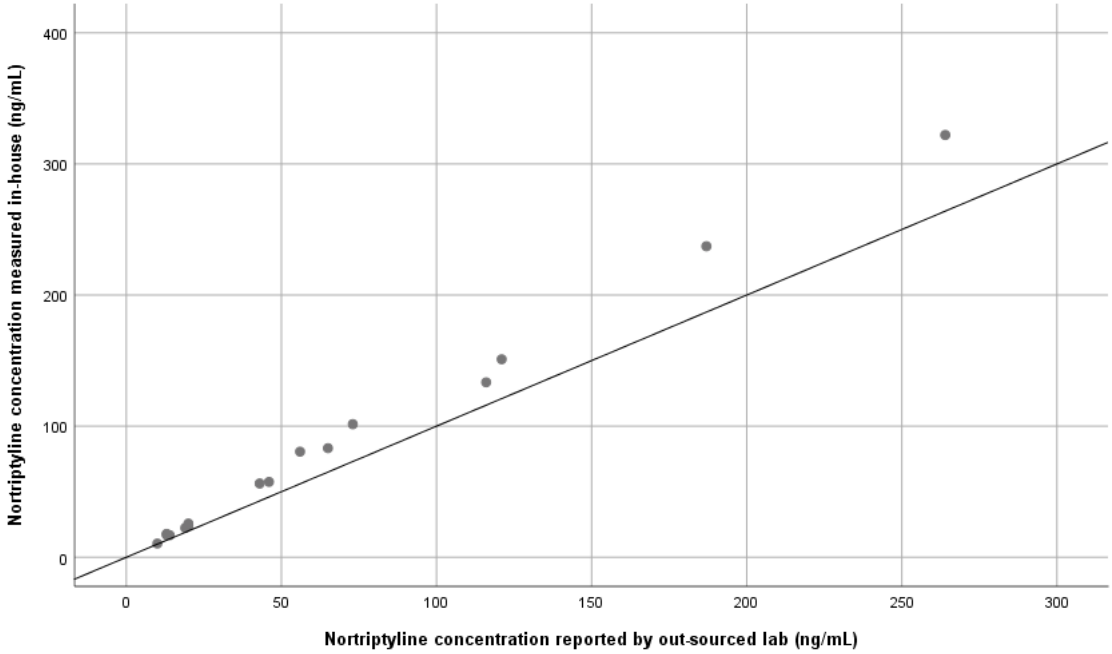


Figure 3-16: Nortriptyline concentrations measured in-house vs reported by out-sourced lab



3.2.3 *CYP2C19* and *CYP2D6* genotypic measures

Genomic DNA extraction and *CYP2D6* and *CYP2C19* genotyping during experimentation at the BioDNA Laboratory, Malta Life Sciences Park, was completed within sessions of around eight hours, with the possibility of running a number of samples per session. At first, several runs had to be repeated because of one or more of the reactions failing. Through refinement of various steps in the procedure, experimentation with the TrimGen Mutector™ genotyping kits progressed from results that were difficult to visualise due to interfering peaks and uninterpretable signals, to results that enable comparison to standards, albeit essentially inconclusive (Appendix B).

Buccal swabs from 44 patients on amitriptyline therapy (recruitment detailed in the clinical sections) were tested at X-Gene Diagnostics, US, to determine *CYP2C19* and *CYP2D6* genotype, including copy-number variation (CNV) analysis for the latter. Table 3-10 details the genotyping and metaboliser status results reported by the lab.

Table 3-10: Lab-reported CYP2C19 and CYP2D6 genotyping results for the recruited patients

Patients genotyped	**CYP2C19**		**CYP2D6**		
PSY – recruited from POP Clinic *PN – recruited from Pain Clinic*	Genotype	Metaboliser Status	Genotype	CNV	Metaboliser Status
PSY 1	**1/*1*	Normal	**2A/*4*	2	Normal
PSY 2	**1/*1*	Normal	**1/*1*	2	Normal
PSY 3	**1/*17*	Rapid	**1/*4*	2	Normal
PSY 4	**1/*9*	Intermediate	**1/*2/xN*	3	Ultra-Rapid
PSY 5	**1/*1*	Normal	**1/*1*	2	Normal
PSY 6	**1/*1*	Normal	**1/*1*	2	Normal
PSY 7	**1/*1*	Normal	**1/*10*	2	Normal
PSY 8	**1/*1*	Normal	*Indeterminate*	2	Unknown
PSY 9	**1/*2*	Intermediate	**2A/*4*	2	Normal
PSY 10	**1/*1*	Normal	**1/*10*	2	Normal
PSY 11	**1/*17*	Rapid	**1/*4*	2	Normal
PSY 12	**1/*1*	Normal	**4/*10*	2	Intermediate
PSY 13	**1/*1*	Normal	**1/*10*	2	Normal
PSY 14	**1/*1*	Normal	**10/*10*	2	Normal
PSY 15	**1/*1*	Normal	**10/*10*	2	Normal
PSY 16	**1/*2*	Intermediate	**1/*41*	2	Normal
PSY 17	**1/*2*	Intermediate	**1/*4/xN*	3	[Ultra-Rapid]
PSY 18	**1/*17*	Rapid	**1/*4*	2	Normal
PSY 19	**1/*1*	Normal	**1/*41*	2	Normal
PSY 20	**1/*1*	Normal	**4/*41*	2	Intermediate
PSY 21	**1/*1*	Normal	**1/*10*	2	Normal
PSY 22	**1/*2*	Intermediate	**1/*4*	2	Normal
PSY 23	**1/*1*	Normal	**10/*10*	2	Normal
PSY 24	**1/*1*	Normal	**10/*10*	2	Normal
PN 1	**1/*2*	Intermediate	**4/*41*	2	Intermediate
PN 2	**1/*2*	Intermediate	**1/*2/xN*	3	Ultra-Rapid
PN 3	**1/*17*	Rapid	**2/*2*	2	Normal
PN 4	/	Failed	/	/	Failed
PN 5	**1/*1*	Normal	**1/*1*	2	Normal
PN 6	**1/*1*	Normal	**1/*4*	2	Normal
PN 7	**1/*1*	Normal	**1/*2A*	2	Normal
PN 8	**1/*1*	Normal	**1/*41*	2	Normal
PN 9	**1/*1*	Normal	**1/*1/xN*	3	Ultra-Rapid
PN 10	**1/*1*	Normal	**1/*1*	2	Normal
PN 11	**1/*1*	Normal	**1/*3*	2	Normal
PN 12	**1/*1*	Normal	**1/*1*	2	Normal
PN 13	**1/*1*	Normal	**1/*1*	2	Normal
PN 14	**1/*1*	Normal	**1/*1*	2	Normal
PN 15	**1/*1*	Normal	**1/*1/xN*	3	Ultra-Rapid
PN 16	**1/*1*	Normal	**1/*4*	2	Normal
PN 17	**2/*17*	Intermediate	**1/*4*	2	Normal
PN 18	**1/*1*	Normal	**1/*1*	2	Normal
PN 19	**1/*17*	Rapid	**2A/*10*	2	Normal
PN 20	**1/*1*	Normal	**1/*1*	2	Normal

The swabs from one patient failed the test due to low DNA concentration; buccal cell sample collection on a second occasion to repeat analysis yielded same result. For another patient, the *CYP2D6* genotype was reported as indeterminate and described by the laboratory as a novel, unknown genotype.

A *CYP2D6* gene copy number of 3 was reported for 5 patients, rendering 4 patients ultra-rapid metabolisers. Incidentally, one duplication case included normal function and no function alleles (**1/*4/xN*). The CYP2D6 metaboliser status for this patient (PSY17) was reported as ultra-rapid by the genotyping laboratory which was thereafter revised to normal metaboliser considering that activity score for the respective scenario lies between 1.0 and 2.0.

The **17* allele, linked to increased enzyme activity, was reported in 5 patients, rendering rapid CYP2C19 metaboliser status. No poor metabolisers were identified in the cohort. Table 3-11 displays the *CYP2D6* activity scores and corresponding metaboliser status when updated in line with the newly proposed rationale and recommendations of the 2019 Consensus on CYP2D6 genotype to phenotype.

Table 3-11: Genotype-inferred *CYP2D6* activity scores and metaboliser status for recruited patients, updated in line with the 2019 Consensus on CYP2D6 genotype to phenotype

Patient	**CYP2D6**				
	Genotype	CNV	Metaboliser Status *reported pre-March 2019*	Activity Score	Metaboliser Status *updated post-March 2019*
PSY 1	**2A/*4*	2	Normal	1	Intermediate
PSY 2	**1/*1*	2	Normal	2	Normal
PSY 3	**1/*4*	2	Normal	1	Intermediate
PSY 4	**1/*2/xN*	3	Ultra-Rapid	3	Ultra-Rapid
PSY 5	**1/*1*	2	Normal	2	Normal
PSY 6	**1/*1*	2	Normal	2	Normal
PSY 7	**1/*10*	2	Normal	1.25	Normal
PSY 8	*Indeterminate*	2	Unknown	/	/
PSY 9	**2A/*4*	2	Normal	1	Intermediate
PSY 10	**1/*10*	2	Normal	1.25	Normal
PSY 11	**1/*4*	2	Normal	1	Intermediate
PSY 12	**4/*10*	2	Intermediate	0.25	Intermediate
PSY 13	**1/*10*	2	Normal	1.25	Normal
PSY 14	**10/*10*	2	Normal	0.5	Intermediate
PSY 15	**10/*10*	2	Normal	0.5	Intermediate
PSY 16	**1/*41*	2	Normal	1.5	Normal
PSY 17	**1/*4/xN*	3	[Normal]	1-2	Intermediate/Normal
PSY 18	**1/*4*	2	Normal	1	Intermediate
PSY 19	**1/*41*	2	Normal	1.5	Normal
PSY 20	**4/*41*	2	Intermediate	0.5	Intermediate
PSY 21	**1/*10*	2	Normal	1.25	Normal
PSY 22	**1/*4*	2	Normal	1	Intermediate
PSY 23	**10/*10*	2	Normal	0.5	Intermediate
PSY 24	**10/*10*	2	Normal	0.5	Intermediate
PN 1	**4/*41*	2	Intermediate	0.5	Intermediate
PN 2	**1/*2/xN*	3	Ultra-Rapid	3	Ultra-Rapid
PN 3	**2/*2*	2	Normal	2	Normal
PN 4	/	/	Failed	/	/
PN 5	**1/*1*	2	Normal	2	Normal
PN 6	**1/*4*	2	Normal	1	Intermediate
PN 7	**1/*2A*	2	Normal	2	Normal
PN 8	**1/*41*	2	Normal	1.5	Normal
PN 9	**1/*1/xN*	3	Ultra-Rapid	3	Ultra-Rapid
PN 10	**1/*1*	2	Normal	2	Normal
PN 11	**1/*3*	2	Normal	1	Intermediate
PN 12	**1/*1*	2	Normal	2	Normal
PN 13	**1/*1*	2	Normal	2	Normal
PN 14	**1/*1*	2	Normal	2	Normal
PN 15	**1/*1/xN*	3	Ultra-Rapid	3	Ultra-Rapid
PN 16	**1/*4*	2	Normal	1	Intermediate
PN 17	**1/*4*	2	Normal	1	Intermediate
PN 18	**1/*1*	2	Normal	2	Normal
PN 19	**2A/*10*	2	Normal	1.25	Normal
PN 20	**1/*1*	2	Normal	2	Normal

3.3 Clinical observations

Results from the clinical investigation are presented as observations from clinical practice in two major therapeutic areas where amitriptyline is prescribed – psychiatry and pain – coupled with the interpretation of outcomes from the analyses carried out within this research for the respective individuals. The latter includes assessment of patient demographics (Section 3.3.1), genotype, metaboliser status and concomitant drugs (Section 3.3.2), serum levels of parent drug and metabolites during amitriptyline therapy (Section 3.3.3), and CYP450 enzymes, serum concentrations and side-effects (Section 3.3.4). The extensive data evaluation, encompassing both *CYP2C19* and *CYP2D6* as genes of interest, estimation of concentration ratios, and consideration of CYP inhibition risk portended by co-administered drugs, curbs confounding factors and facilitates understanding of practical implications concerned with genotype-guided dosing recommendations.

3.3.1 Patient population demographics

A total of forty-four (44) patients were recruited from the two enrolling arms of the research: twenty-four (24) patients from the Psychiatric Outpatients (POP) Clinic, and twenty (20) patients from the Pain Clinic, at Mater Dei Hospital. Overall, the cohort consisted of 32 females and 12 males, with a mean age of 58 ±15 years (median 61, range 24 – 79) and mean weight of 79 ±19 Kg (median 78, range 53 – 158). Routine bloods identified patients with altered levels of bilirubin (1), alkaline phosphatase (7), GGT (6), ALT (6), urea (2), and/or creatinine (7). Most individuals were married (64%), non-smokers (68%) and did not consume alcohol regularly (98%). The majority of patients

were unemployed (60% of patients recruited from pain clinic and 83% of patients recruited from POP respectively).

All recruited subjects reported a time since onset of over 1 year for the underlying condition being presently treated with amitriptyline. The pain scores reported by the pain subjects (as an average intensity over the 4 weeks prior to assessment, scored on a scale from 0 to a maximum of 10 on PainDETECT) varied between 3 and 10 (median of 7) between subjects, with the presence of a neuropathic pain component being mostly ambiguous verging on the unlikely. MADRS scores (which may range from 0 to 60 with increasing depression severity) were on average 18 amongst the psychiatric group, with a standard deviation of 13. Past hospitalisations related to the respective condition were recounted by fewer patients under pain management (2 out of 20) compared to patients under psychiatric care (7 out of 24). A relevant family history was reported more frequently in the psychiatry group (10 out of 24) than in the pain group (4 out of 20).

3.3.2 Genotype, metaboliser status and concomitant drugs

The tested cohort consisted of 44 patients. Genotyping results with consequent metaboliser status, were available for *CYP2C19* in 43 patients (1 failed), and for *CYP2D6* in 42 patients (1 failed, 1 indeterminate). Aberrant metabolism for CYP2C19 or CYP2D6 was respectively identified in 30% and 17% of the patients for whom a conclusive result for the tests was made available by the laboratory, with 3 of these patients reported to have irregular metabolism at both CYP2C19 and CYP2D6. Consequences of the alleles identified in the sample, on activity of CYP2C19 and CYP2D6, are explained in Table 3-12. In Table 3-13 and Figure 3-17, an overview of the corresponding genotypic categories characterised by the laboratory-reported genotyping is provided. The square brackets []

highlight presentations that may be considered as intermediate metabolisers, as per the 2019 Consensus on genotype to phenotype. Updating the CYP2D6 metaboliser status in line with the 2019 Consensus renders 50% of patients to potentially deviate from the normal CYP2D6 metaboliser status. As deliberated during the August 2019 CPIC conference call[59], the impact of changes to activity score and phenotypes is most evident in CYP2D6 intermediate metabolisers, which more than double in number, with the CPIC guidelines for tricyclic antidepressants being most affected.

Table 3-12: Distribution of alleles in recruited patients, and corresponding activity

Gene	**Activity**	**Allele**	**Number in sample**	**Gene**	**Activity**	**Allele**	**Number in sample**
CYP2C19	None	**2*	7	*CYP2D6*	None	**3*	1
						**4*	13
	Decreased	**9*	1		Decreased	**10*	14
						**41*	5
	Normal	**1*	72		Normal	**1*	43
						**2*	4
						**2A*	4
	Increased	**17*	6			*xN*	5

Table 3-13: CYP2C19 and CYP2D6 metaboliser status characterised in sample population

Characterisation	**CYP2C19**	
	Presentation	Number (%) in sample
Intermediate metaboliser	**1/*2, *2/*17, *1/*9*	8 (18%)
Normal metaboliser	**1/*1*	30 (70%)
Rapid metaboliser	**1/*17*	5 (12%)
Characterisation	**CYP2D6**	
	Presentation	Number (%) in sample
Intermediate metaboliser	**4/*41, *4/*10*	3 (7%)
Normal metaboliser	**1/*1, [*1/*4], *1/*10, [*10/*10], *1/*41, [*2A/*4], *1/*2A, [*1/*3], *2/*2, *2A/*10, [*1/*4/xN]*	35 (83%)
Ultra-Rapid metaboliser	**1/*1/xN, *1/*2/xN*	4 (10%)

[59] Clinical Pharmacogenetics Implementation Consortium. Minutes CPIC Conference Call; August 1, 2019. Available from the CPIC member site: https://cpicpgx.org/2019-cpic-conference-call-minutes/.

Figure 3-17: Distribution of genotypic categories in sample population

Genotype should be considered along with patient characteristics and potential drug interactions. Patients treated for pain, and especially psychiatric disorders, often require multiple medications, which may cause conversion to a poor metaboliser status. Patients recruited from the Pain Clinic, on average were receiving 2 concomitant drugs, whereas patients recruited from the POP Clinic had on average 5 drugs co-administered with amitriptyline. Three out of the twenty patients under pain management were being prescribed one or more drugs that may affect CYP2C19 and/or CYP2D6. This was

conceivably higher in patients under psychiatric care whose treatment plan included a drug with potential CYP impact in 18 out of the 24 patients.

Paroxetine was co-administered in 11 cases, followed by omeprazole appearing in 8 cases, ranitidine (3), chlorpromazine (2), and citalopram, escitalopram, fluvoxamine and venlafaxine recorded in 1 patient each. Among other concomitant drugs were the CYP2C19 substrates rabeprazole and diazepam. Since interactions that may arise at CYP level could shift concentrations of amitriptyline and its metabolites in blood, the risk of inhibition by concomitant drugs was considered in subsequent analyses.

3.3.3 Serum levels of parent drug and metabolites during amitriptyline therapy

The amitriptyline dose administered in the recruited subjects ranged between 10mg and 175mg per day. The data – daily amitriptyline doses and measured concentrations – was not normally distributed (Shapiro-Wilk Test, P=0.000) and correlations were tested with the non-parametric Spearman test. Table 3-14 explains the patient sample included in the analyses.

Table 3-14: Patient sample included in evaluation of serum levels

N = 44 patients *24 subjects recruited for POP and 20 subjects recruited from Pain Clinic, MDH*
n = 42 patients *excluded 2 subjects having blood withdrawn at 4-hours post dose* → Analysis of daily dose vs measured concentrations, considering weight, age and gender
n = 33 patients *further excluded 2 subjects with inconclusive genotyping results, and 7 subjects whose daily amitriptyline dosage regimen entailed unequal dose distribution* → Analysis of measured concentrations, and ratios, considering CYP2C19 and CYP2D6 → Analysis of Ct & Cmin levels, and expected ranges, considering CYP2C19 and CYP2D6

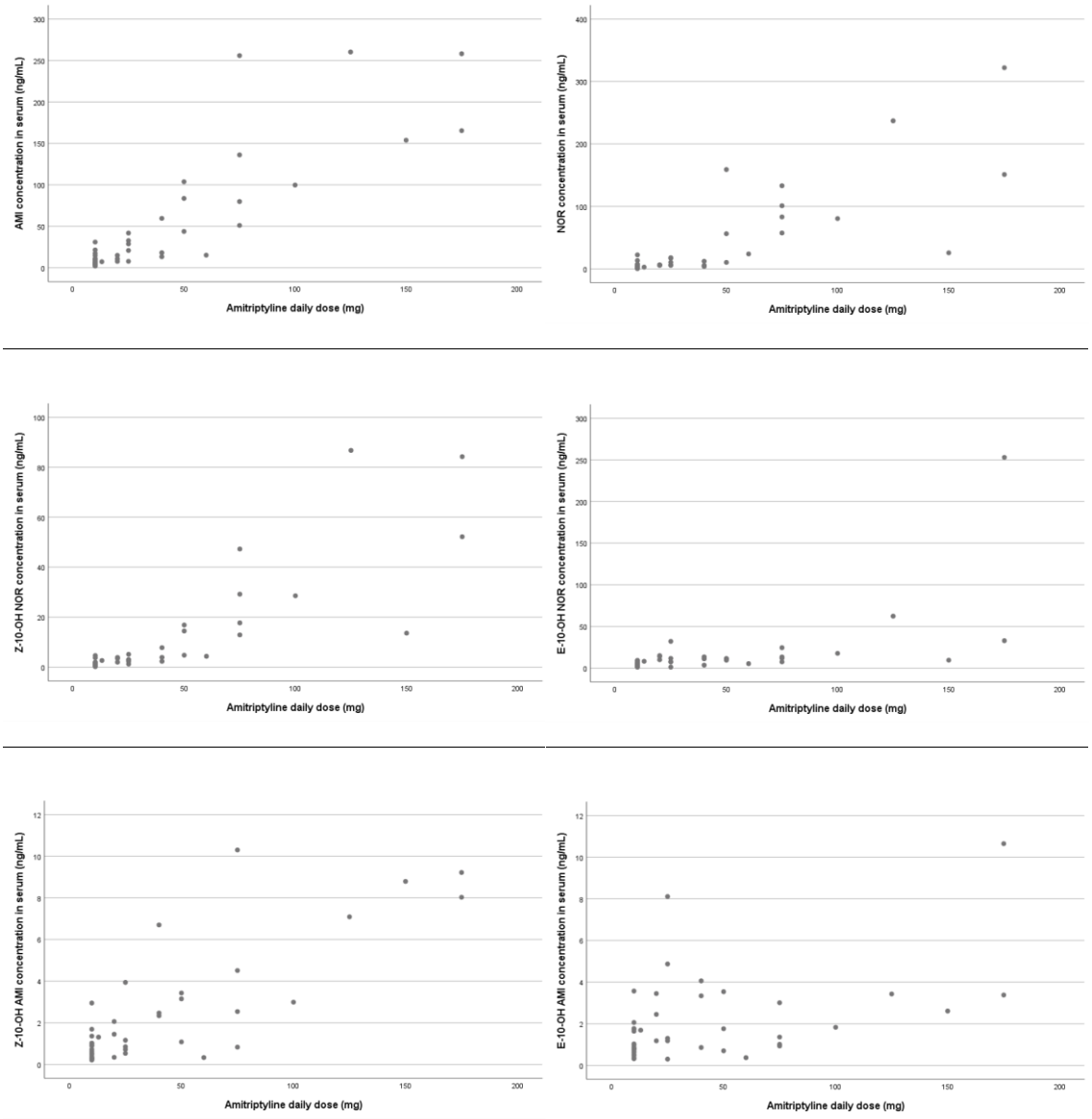
All measured serum concentrations – amitriptyline, nortriptyline, Z-10-OH nortriptyline, E-10-OH nortriptyline, Z-10-OH amitriptyline and E-10-OH amitriptyline – were positively correlated to the daily dose of amitriptyline administered in the respective patient (correlation coefficients: 0.838, 0.820, 0.876, 0.710, 0.679, 0.434, respectively). The correlations were significant at the 0.01 level (Table 3-15, Figure 3-18). Considering patient weight in the analysis (dose included as mg/Kg/day) yielded analogous results, with the significant positive correlation between the daily dose of amitriptyline per bodyweight and serum levels of AMI, NOR, Z-10-OH NOR, E-10-OH NOR, Z-10-OH AMI, and E-10-OH AMI, being upheld (correlation coefficients 0.732, 0.779. 0.821, 0.710, 0.572, 0.371, respectively; $P < 0.05$).

Concentrations of amitriptyline and metabolites were unrelated to age and no significant differences were observed between males (11) and females (31) in the 42-patient sample. Patient age and gender were not considered further in the analyses, with published studies on small cohorts reporting sporadic or absent correlations (Edelbroek et al, 1984, Dahl et al, 1996, Morita, et al 2000). The total of amitriptyline and nortriptyline concentration was observed to be over 350 ng/mL in three patients, one of whom additionally had the highest concentration measured in the patients under study for E-10 hydroxynortriptyline (253 ng/mL). These three patients, on a daily amitriptyline dose of 75 – 175 mg, were determined to be intermediate CYP2C19 metabolisers, as per the genotyping result, with two of them also being administered concomitant CYP inhibitors.

Table 3-15: Test of correlation between amitriptyline daily dose and serum concentrations of parent compound, and its desmethylated and hydroxylated metabolites

Spearman Correlation		AMI	NOR	Z-10-OH NOR	E-10-OH NOR	Z-10-OH AMI	E-10-OH AMI
Amitriptyline daily dose	Correlation Coefficient	0.838	0.820	0.876	0.710	0.679	0.434
	P-value	0.000	0.000	0.000	0.000	0.000	0.004

Figure 3-18: Correlation between the daily dose of amitriptyline and serum levels of amitriptyline and metabolites (AMI, NOR, E-10-OH AMI, Z-10-OH AMI, E-10-OH NOR, and Z-10-OH NOR)



Within the 33-participant cohort included in subsequent analysis, the lab-reported CYP2D6 metaboliser status, as per genotype, was normal (27), intermediate (3) or ultra-rapid (3), while the lab-reported CYP2C19 metaboliser status, as per genotype, was normal (24), intermediate (4) or rapid (5). Concomitant drugs were reviewed in the 33 patients proceeding in the data analysis. Patients on paroxetine were considered at high-risk of CYP2D6 inhibition (7 cases), while escitalopram, citalopram, ranitidine, and

chlorpromazine denoted weak-moderate risk of CYP2D6 inhibition (4 cases). Omeprazole was considered to infer a weak-moderate risk of CYP2C19 inhibition (5 cases). Patients at risk of CYP2D6 inhibition were normal CYP2D6 metabolisers according to genotype, except for one patient for whom intermediate metabolism was reported by the lab. Patients at risk of CYP2C19 inhibition were normal CYP2C19 metabolisers according to genotype, except for two patients for whom rapid metabolism was reported by the lab.

The nortriptyline to amitriptyline concentration ratio ranged between 0.1 and 2.0, with a median of 0.5, and was unrelated to the daily dose. The parsimonious generalised linear model for the nortriptyline to amitriptyline concentration ratio consisted of the CYP2C19 metaboliser status as significant main effect (Table 3-16). The predictors - CYP2D6 metaboliser status, risk of CYP2D6 inhibition by concomitant drugs, and risk of CYP2C19 inhibition by concomitant drugs - were excluded sequentially from the model fit, based on the significance of their contribution. Parameter estimates indicate that the mean nortriptyline to amitriptyline ratio is 0.234 lower in intermediate CYP2C19 metabolisers and 0.579 higher in rapid CYP2C19 metabolisers, compared to normal metabolisers (Figure 3-19).

Table 3-16: Generalised Linear Model – nortriptyline to amitriptyline concentration ratio

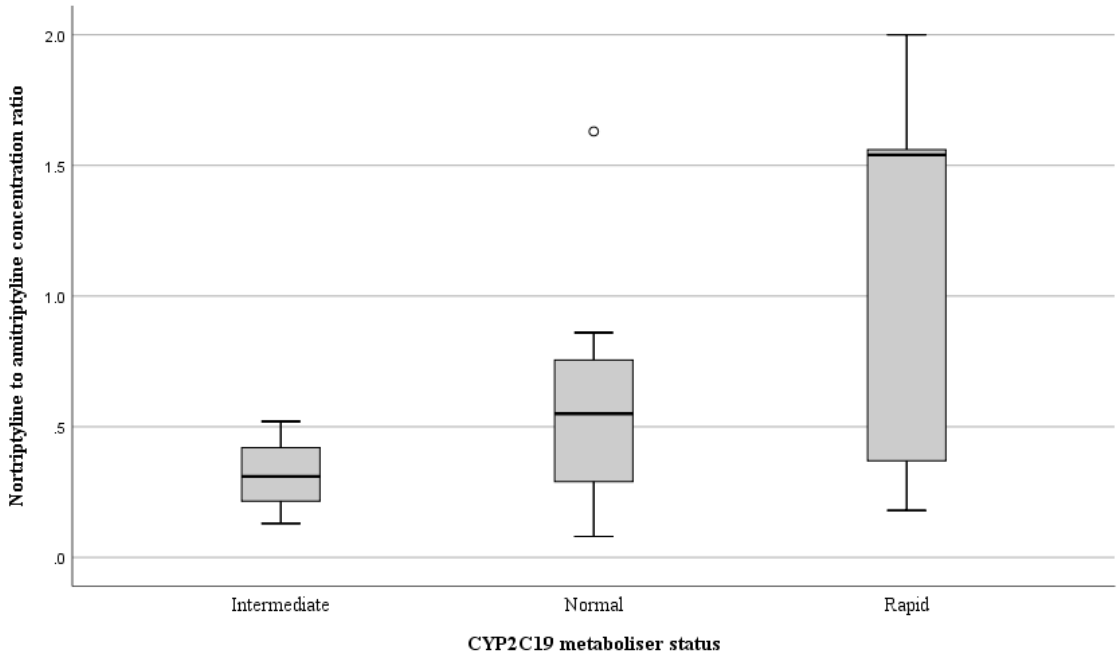
Tests of Model Effects

	Type III		
Source	Wald Chi-Square	df	P-value
(Intercept)	32.614	1	0.000
CYP2C19 Metaboliser Status	7.371	2	0.025

Parameter Estimates

			Hypothesis Test		
Parameter	B	Std. Error	Wald Chi-Square	df	P-value
(Intercept)	0.551	0.073	57.455	1	0.000
CYP2C19 – Rapid	0.579	0.335	2.992	1	0.084
CYP2C19 – Intermediate	-0.234	0.126	3.455	1	0.063
CYP2C19 – Normal	0	.	.	.	.
(Scale)	0.418	0.097			

Figure 3-19: Nortriptyline to amitriptyline concentration ratio as related to CYP2C19



The concentrations of Z-10-OH nortriptyline and Z-10-OH amitriptyline at time of blood withdrawal, normalised to the daily dose, were significantly correlated to the concentration of amitriptyline (Ct, dose-normalised). The concentrations of Z-10-OH nortriptyline and E-10-OH amitriptyline were significantly correlated to the concentration of nortriptyline. The concentration of E-10-OH nortriptyline was not correlated to either amitriptyline or nortriptyline concentration (Table 3-17). Descriptive statistics indicate that the E-10-OH nortriptyline concentration had the highest mean, compared to the other hydroxy-metabolites, among the 33 patients.

The parsimonious generalised linear model for the ratio of hydroxy-metabolites to parent consisted of the CYP2D6 inhibition risk by concomitant drugs (Table 3-18, Table 3-19). The predictors - risk of CYP2C19 inhibition by concomitant drugs, CYP2D6 metaboliser status, and CYP2C19 metaboliser status - were excluded sequentially from the model fit, based on the significance of their contribution. Parameter estimates indicate that the mean ratio of hydroxy-amitriptyline (Z-10-OH AMI + E-10-OH AMI) to amitriptyline and the

mean ratio of hydroxy-nortriptyline (Z-10-OH NOR + E-10-OH NOR) to nortriptyline are lower in patients at high risk of CYP2D6 inhibition by concomitant drugs, compared to patients for whom the risk is none or weak-moderate.

Table 3-17: Concentrations of hydroxy-metabolites – descriptive statistics and correlations

Dose-normalised concentrations	*Mean*	*Std. Deviation*	*Median*	*Minimum*	*Maximum*
Z-10-OH Nortriptyline (Z-10-OH NOR)	0.174	0.129	0.12	0.02	0.63
E-10-OH Nortriptyline (E-10-OH NOR)	0.403	0.280	0.33	0.05	1.28
Z-10-OH Amitriptyline (Z-10-OH AMI)	0.074	0.063	0.06	0.01	0.30
E-10-OH Amitriptyline (E-10-OH AMI)	0.094	0.084	0.07	0.01	0.36

Spearman correlation		*Z-10-OH NOR*	*E-10-OH NOR*	*Z-10-OH AMI*	*E-10-OH AMI*
Dose-normalised amitriptyline Ct	Correlation Coefficient	0.602	-0.202	0.521	0.062
	P-value	0.000	0.259	0.002	0.730
Dose-normalised nortriptyline Ct	Correlation Coefficient	0.726	-0.146	-0.255	-0.490
	P-value	0.000	0.418	0.152	0.004

Table 3-18: Generalised Linear Model – hydroxy-amitriptyline to amitriptyline ratio

Tests of Model Effects

	Type III		
Source	Wald Chi-Square	df	P-value
(Intercept)	59.965	1	0.000
Concomitant CYP2D6 inhibitor risk	63.531	2	0.000

Parameter Estimates

			Hypothesis Test		
Parameter	B	Std. Error	Wald Chi-Square	df	P-value
(Intercept)	0.040	0.008	26.874	1	0.000
CYP2D6 inhibition risk – None	0.205	0.028	54.507	1	0.000
CYP2D6 inhibition risk – Weak/Moderate	0.205	0.063	10.590	1	0.001
CYP2D6 inhibition risk – High	0	.	.	.	.
(Scale)	0.260	0.062			

Table 3-19: Generalised Linear Model – hydroxy-nortriptyline to nortriptyline ratio

Tests of Model Effects

Source	Type III Wald Chi-Square	df	P-value
(Intercept)	48.265	1	0.000
Concomitant CYP2D6 inhibitor risk	49.463	2	0.000

Parameter Estimates

Parameter	B	Std. Error	Hypothesis Test Wald Chi-Square	df	P-value
(Intercept)	0.363	0.080	20.728	1	0.000
CYP2D6 inhibition risk – None	1.914	0.293	42.636	1	0.000
CYP2D6 inhibition risk – Weak/Moderate	1.782	0.628	8.045	1	0.005
CYP2D6 inhibition risk – High	0	.	.	.	.
(Scale)	0.338	0.079			

The expected dose-related reference concentration range for amitriptyline and nortriptyline, at the respective time of blood withdrawal (Ct), was calculated for the 33 patients in the sample. Twelve out of 33 patients (36%) fit within the predicted amitriptyline Ct range, while 21 patients (64%) had their measured concentrations above (16) or below (5) the range. Thirteen patients (39%) fit within the predicted nortriptyline range, while 20 patients (61%) had their measured concentrations above (8) or below (12) the range. In patients at high risk of CYP2D6 inhibition by concomitant drugs, the amitriptyline + nortriptyline Ct was on average 52% above the higher end of the expected concentration range (Kruskal Wallis test, P=0.001; Table 3-20). The likelihood of a patient's amitriptyline + nortriptyline Ct being above the expected range was observed to increase with increased risk of CYP2D6 inhibition by concomitant drugs. The parsimonious logistic regression model (Table 3-21) identified one predictor – risk of CYP2D6 inhibition by concomitant drug(s) – which explained 44.3% of the total variation in measured concentrations of amitriptyline + nortriptyline being below, within, or above the expected Ct range (P=0.003).

Table 3-20: Difference in measured Ct from expected amitriptyline + nortriptyline range, according to risk of CYP2D6 inhibition by concomitant drug(s)

CYP2D6 inhibition risk	Sample Size	Mean % Difference from expected Ct range	Std. Deviation	P-value	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
None	22	-2.663	28.886	0.001	0.000	10.144
Weak/Moderate	4	1.818	3.635		0.000	7.602
High	7	51.684	25.997		27.641	75.728

The odds that a patient with no CYP2D6 inhibition risk lies below the expected amitriptyline + nortriptyline range rather than above the range is 570289643 times that of a patient with high inhibition risk. The odds that a patient with weak-moderate CYP2D6 inhibition risk lies below the expected range rather than above, is 5.064 times that of a patient with high inhibition risk. The odds that a patient with no CYP2D6 inhibition risk lies within the expected amitriptyline + nortriptyline range, rather than above, is 427717240 times that of a patient with high inhibition risk. The odds that a patient with weak-moderate CYP2D6 inhibition risk lies within the expected amitriptyline + nortriptyline range rather than above the range is 998006895 times that of a patient with high inhibition risk.

Analysis so far considered concentrations at time of blood withdrawal (Ct). Thereafter, trough concentrations (Cmin) were estimated from the measured concentrations (Ct) for the 33 patients. Expected trough dose-related reference ranges (Cmin) were calculated for amitriptyline, nortriptyline, and amitriptyline + nortriptyline, to identify patients outside the predicted ranges. The results are analogous to the previous Ct considerations with respect to patients within or outside the ranges (Table 3-22). To enable direct comparison between subjects, Cmin concentrations, normalised to the dosing schedule for the 33 patients under study, were used in investigating between-patient variability in

concentrations and the influence of metaboliser status and CYP inhibition risk of co-medication on steady-state Cmin.

Table 3-21: Logistic regression model – Measured Ct being below/within/above the expected amitriptyline + nortriptyline range

	Model Fitting Criteria	Likelihood Ratio Tests		
Effect	-2 Log Likelihood of Reduced Model	Chi-Square	df	P-value
Intercept	50.435			
Concomitant CYP2D6 inhibitor risk	34.256	16.178	4	0.003

Parameter estimates

Ct Expected Range for amitriptyline + nortriptyline[a]		B	Std. Error	Wald	df	Sig.	Odds Ratio
Below	Intercept	-20.316	.556	1333.442	1	0.000	
	CYP2D6 inhibition risk *None*	20.162	0.000	.	1	.	570289643.772
	CYP2D6 inhibition risk *Weak/Moderate*	1.622	0.000	.	1	.	5.064
	CYP2D6 inhibition risk *High*	0	.	.	0	.	.
Within	Intercept	-19.623	6893.776	0.000	1	0.998	
	CYP2D6 inhibition risk *None*	19.874	6893.776	0.000	1	0.998	427717240.889
	CYP2D6 inhibition risk *Weak/Moderate*	20.721	6893.776	0.000	1	0.998	998006895.408
	CYP2D6 inhibition risk *High*	0	.	.	0	.	.

a. The reference category is: Above Ct Expected Range.

Table 3-22: Cmin of patients, as compared to expected range

Cmin (N = 33 patients)	Amitriptyline	Nortriptyline	Amitriptyline + Nortriptyline
Below expected range	5 (15%)	12 (36%)	6 (18%)
Within expected range	12 (36%)	13 (39%)	12 (36%)
Above expected range	16 (48%)	8 (24%)	15 (45%)

The Kruskal Wallis test statistics showed no significant relationship between normalised amitriptyline Cmin concentrations and lab-reported CYP2D6 or CYP2C19 metaboliser status, as determined by genotype, or CYP2C19 inhibition risk by concomitant drugs (P = 0.485, 0.324, 0.248, respectively). A significant correlation was identified between normalised amitriptyline Cmin concentrations and CYP2D6 inhibition risk by concomitant drugs (none/weak-moderate/high) with a P-value of 0.005. When the CYP2D6 inhibition risk is categorized into 2 groups (rather than 3), i.e. none-weak-moderate / high, the correlation becomes significant at the 0.01 level (Table 3-23). The average normalised amitriptyline Cmin was 1.5 ng/mL in patients at high risk of CYP2D6 inhibition by concomitant drugs, compared to 0.6 ng/mL in patients where the risk is conceivably lower.

A similar scenario was replicated for the normalised nortriptyline Cmin concentrations, with Kruskal Wallis results as follows: CYP2C19 metaboliser status (P=0.781), CYP2D6 metaboliser status (P=0.320), risk of CYP2C19 inhibition by concomitant drugs (P=0.292). A significant relationship was noted between normalised nortriptyline Cmin concentrations and risk of CYP2D6 inhibition by concomitant drugs (P=0.001), which became stronger upon 2-group categorisation as previously described (Table 3-24). The average normalised nortriptyline Cmin was 1.3 ng/mL in patients at high risk of CYP2D6 inhibition by concomitant drugs, compared to 0.3 ng/mL in patients where the risk is conceivably lower.

Table 3-23: Relation between normalised amitriptyline Cmin concentration and risk of CYP2D6 inhibition by concomitant drug(s)

CYP2D6 inhibition risk	Sample Size	Mean Normalised Amitriptyline Cmin	Std. Deviation	P-value	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
None-Weak-Moderate	26	0.585	0.3298	0.002	0.452	0.718
High	7	1.499	0.7210		0.832	2.166

Table 3-24: Relation between normalised nortriptyline Cmin concentration and risk of CYP2D6 inhibition by concomitant drug(s)

CYP2D6 inhibition risk	Sample Size	Mean Normalised Nortriptyline Cmin	Std. Deviation	P-value	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
None-Weak-Moderate	26	0.284	0.2162	0.000	0.197	0.372
High	7	1.293	0.8319		0.524	2.062

Analogous results were obtained for the normalised amitriptyline + nortriptyline Cmin concentration with CYP2C19 metaboliser status, CYP2D6 metaboliser status, risk of CYP2C19 inhibition by concomitant drugs, and risk of CYP2D6 inhibition by concomitant drugs (P = 0.504, 0.288, 0.120, 0.001 respectively). When CYP2D6 inhibition is categorized into 2 groups, a P-value of 0.000 outlines its strong significant relationship with the normalised amitriptyline + nortriptyline Cmin concentration which is on average 0.9 ng/mL in patients at low risk, increasing to 2.8 ng/mL at high risk of CYP2D6 inhibition (Table 3-25).

Table 3-25: Relation between normalised amitriptyline + nortriptyline Cmin concentration and risk of CYP2D6 inhibition by concomitant drug(s)

CYP2D6 inhibition risk	Sample Size	Mean Normalised AMI + NOR Cmin	Std. Deviation	P-value	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
None-Weak-Moderate	26	0.869	0.4226	0.000	0.699	1.040
High	7	2.792	1.4263		1.473	4.111

The correlation results were sustained in the generalized linear models for the Cmin concentrations of amitriptyline, nortriptyline, and amitriptyline + nortriptyline, with the risk of CYP2D6 inhibition by concomitant drug(s) being the lone predictor within the parsimonious models. Parameter estimates indicate that normalised Cmin concentrations of amitriptyline, nortriptyline, and amitriptyline + nortriptyline, are higher in patients at higher risk of CYP2D6 inhibition by concomitant drugs (Table 3-26).

Table 3-26: Generalized linear model – Normalised Cmin amitriptyline, nortriptyline and amitriptyline + nortriptyline

Amitriptyline normalised Cmin

Tests of Model Effects

Source	Type III Wald Chi-Square	df	P-value
(Intercept)	45.822	1	0.000
Concomitant CYP2D6 inhibitor risk	8.814	1	0.003

Parameter Estimates

Parameter	B	Std. Error	Hypothesis Test Wald Chi-Square	df	P-value
(Intercept)	1.499	0.302	24.680	1	0.000
CYP2D6 inhibition risk: None-Weak-Moderate	-0.914	0.308	8.814	1	0.003
CYP2D6 inhibition risk: High	0	.	.	.	.
(Scale)	0.284	0.067			

Nortriptyline normalised Cmin

Tests of Model Effects

Source	Type III Wald Chi-Square	df	P-value
(Intercept)	23.975	1	0.000
Concomitant CYP2D6 inhibitor risk	9.809	1	0.002

Parameter Estimates

Parameter	B	Std. Error	Hypothesis Test Wald Chi-Square	df	P-value
(Intercept)	1.293	0.320	16.323	1	0.000
CYP2D6 inhibition risk: None-Weak-Moderate	-1.009	0.322	9.809	1	0.002
CYP2D6 inhibition risk: High	0	.	.	.	.
(Scale)	0.429	0.099			

Amitriptyline + Nortriptyline normalised Cmin

Tests of Model Effects

Source	Type III Wald Chi-Square	df	P-value
(Intercept)	49.683	1	0.000
Concomitant CYP2D6 inhibitor risk	13.703	1	0.000

Parameter Estimates

Parameter	B	Std. Error	Hypothesis Test Wald Chi-Square	df	P-value
(Intercept)	2.792	0.513	29.646	1	0.000
CYP2D6 inhibition risk: None-Weak-Moderate	-1.923	0.520	13.703	1	0.000
CYP2D6 inhibition risk: High	0	.	.	.	.
(Scale)	0.236	0.056			

In view of the significant contribution observed with respect to the risk of CYP2D6 inhibition by concomitant drugs, an exploratory exercise was carried out in attempt of explaining the variation in normalised Cmin concentrations, which emerged to be more related to this risk, rather than to the lab-reported CYP2D6 metaboliser status determined by the genotype. The CYP2D6 metaboliser status for the patients at high risk of CYP2D6 inhibition (6 reported to be normal metabolisers and one intermediate metaboliser for CYP2D6 in genotyping results) was switched to 'intermediate' for all 7 patients.

The relationship between CYP2D6 metaboliser status to amitriptyline, nortriptyline, and amitriptyline + nortriptyline normalised Cmin, which was previously reported as not significant, became significant when the CYP2D6 metaboliser status for these 7 patients was revised in the analysis (P = 0.015, 0.002, 0.004, respectively; Table 3-27). The mean normalised amitriptyline and nortriptyline Cmin concentrations increase respectively from 0.4 and 0.2 ng/mL in ultra-rapid metabolisers to 0.6 and 0.3 ng/mL in normal metabolisers to 1.3 and 1.1 ng/mL in intermediate metabolisers.

Table 3-27: Relation between normalised amitriptyline + nortriptyline Cmin concentration and revised CYP2D6 status

CYP2D6 metaboliser status ***following revision***	Sample Size	Mean Normalised AMI + NOR Cmin	Std. Deviation	P-value	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Ultra-Rapid	3	0.644	0.457		0.000	1.779
Normal	21	0.875	0.369	0.004	0.708	1.043
Intermediate	9	2.426	1.477		1.290	3.561

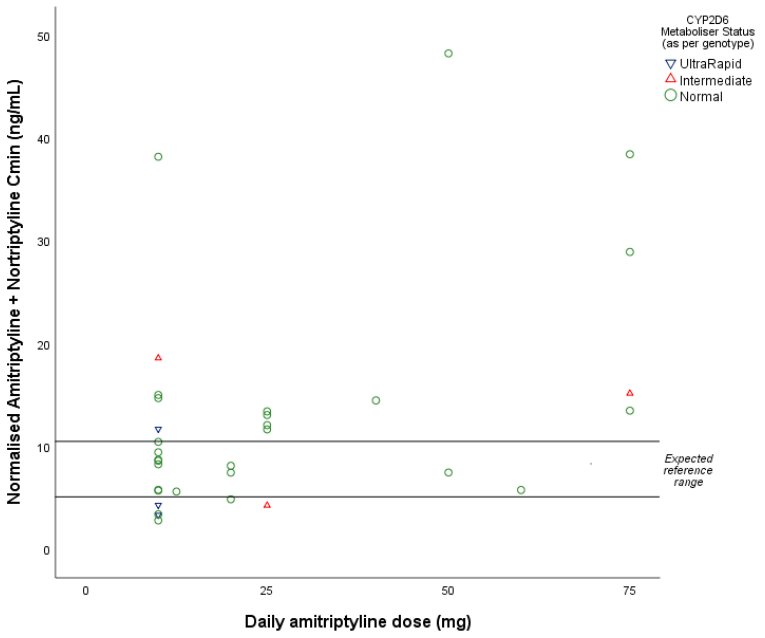
Figure 3-20 depicts a scenario wherein the amitriptyline + nortriptyline Cmin for all 33 patients was normalised to a 10mg once daily dose, in which case the expected dose-

related reference range is 5.1 – 10.5 ng/mL (indicated by the horizontal lines). Figure 3-20 (A.) shows that an intermediate lab-reported CYP2D6 metaboliser status, as determined by genotype, could explain 2 out of the 15 patients above the expected range (red marker). Figure 3-20 (B.) shows that a high risk of CYP2D6 inhibition by concomitant drug could explain 7 out of the 15 patients above the expected range, particularly those where difference from the expected is considerable (red marker). Figure 3-20 (C.) shows that the CYP2D6 metaboliser status, revised in cases of high inhibition risk, could explain 8 out of the 15 patients above the range whereby the elevated Cmin is potentially linked to an intermediate (or possibly poor) CYP2D6 metaboliser status which is either genetically determined or provoked by a concomitant drug (red marker).

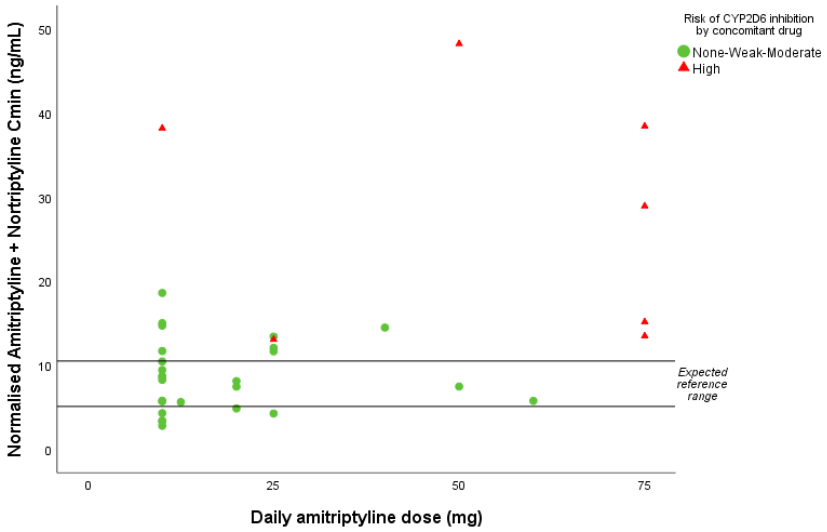
The analyses detailed in this section (3.3.3) were repeated, replacing the lab-reported CYP2D6 metaboliser status with the *CYP2D6* activity score or an 'updated' CYP2D6 metaboliser status, in line with the latest CPIC consensus (as per Table 3-11). The results were largely analogous, with no correlation observed between the 'updated' CYP2D6 metaboliser status or *CYP2D6* activity score, and the serum levels under study, with risk of inhibition by concomitant CYP inhibitors also persisting as significant lone predictor in the modelling outcomes for concentrations being below/within/above expected ranges. Conversely, a significant positive relationship was observed between *CYP2D6* activity score and the ratio of E-10-OH amitriptyline + Z-10-OH amitriptyline to amitriptyline, as well as the ratio of E-10-OH nortriptyline + Z-10-OH nortriptyline to nortriptyline (R=0.385, P=0.027; R=0.369, P=0.034; Figure 3-21, A and B; respectively). The ratio of hydroxy-metabolites to parent increases with higher *CYP2D6* activity scores.

Figure 3-20: Variation in amitriptyline + nortriptyline normalised Cmin among patients

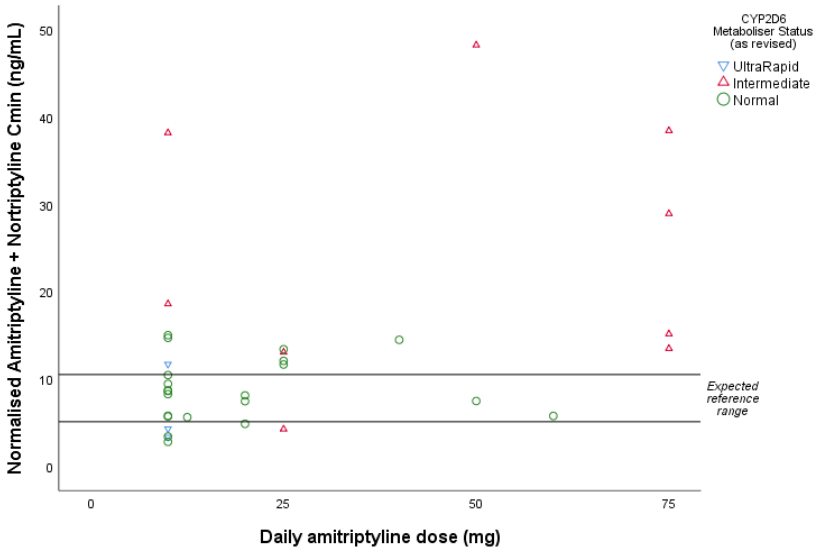
A.



B.



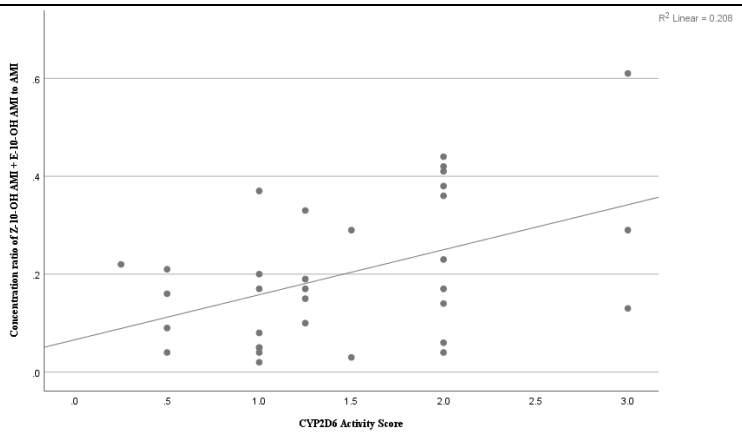
C.



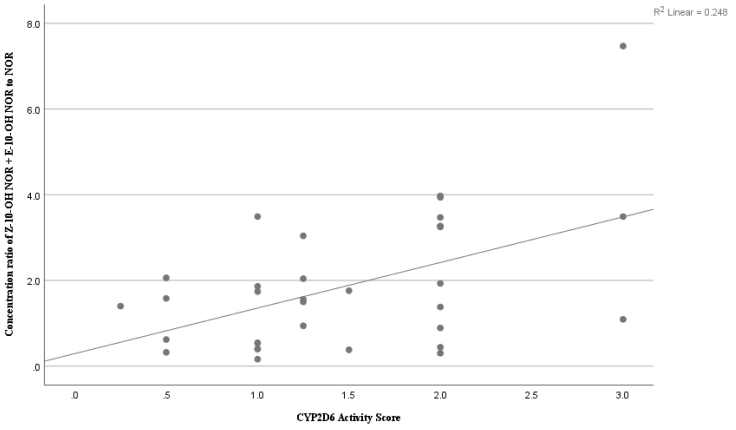
As for the outcomes depicted by Figure 3-20, the 'updated' CYP2D6 metaboliser status would explain 7 out of 15 patients above the expected amitriptyline + nortriptyline concentration range (compared to 2 for the lab-reported CYP2D6 metaboliser status). A 'revised and updated' metaboliser status, i.e. switching CYP2D6 metaboliser status to 'intermediate' in line with CPIC consensus and if risk of CYP2D6 inhibition is high, would provide an explanation for 10 patients. The remaining 5 patients with concentrations above the expected range include 1 CYP2D6 ultra-rapid and 1 CYP2C19 rapid metaboliser, 2 patients being co-administered a CYP2C19 inhibitor, and 1 patient being co-administered a weak-moderate CYP2D6 inhibitor. Essentially, the amitriptyline + nortriptyline levels in these 5 patients are close to the upper limit of the range.

Figure 3-21: Correlations between hydroxy-metabolites to parent concentration ratios and *CYP2D6* activity score

A.



B.



In the 33-patient cohort, the amitriptyline + nortriptyline concentration - Cmin normalised to a 10mg once daily dose - ranged between: 2.8 and 48.2 ng/mL. Genotype-guided dosing recommendations were anticipated to be most relevant for psychiatry patients, in view of the higher amitriptyline doses, and higher co-administration of CYP inhibitors. Out of the 17 psychiatry patients in the 33-patient cohort, 7 subjects were considered likely CYP2C19 and CYP2D6 normal metabolisers, in line with genotype results interpreted as per the 2019 Consensus of genotype to phenotype. The amitriptyline + nortriptyline concentration, still ranged considerably in these 7 patients, between: 8.12 and 38.17 ng/mL. Table 3-28 presents a concise assessment of the 17 patients recruited from POP, including annotations based on the CPIC guideline for tricyclic antidepressants and CYP2D6 and CYP2C19 metaboliser status[60], as well as evaluation informed by the data collection and analyses carried out.

Outcomes point towards an intertwined scenario implying that serum concentrations might not be predictable by genotyping alone. The potential of phenoconversion, whereby metaboliser status may be altered through drug interactions, represents a concern that may be underserved by present algorithms for explaining clinical implications.

[60] PharmGKB. Annotation of CPIC Guideline for amitriptyline and CYP2C19, CYP2D6 [Online]. PharmGKB [accessed 2019 Jun 12]. Available from: https://www.pharmgkb.org/guidelineAnnotation/PA166105006.

Table 3-28: Genotype-guided dosing recommendations, considering serum levels and concomitant CYP2C19/CYP2D6 inhibitors, for recruited patients under psychiatric care

Patient	**CYP2C19**		**CYP2D6**		
	Genotype	Metaboliser Status	Genotype	Activity Score	Metaboliser Status *updated post-March 2019*
PSY 5	**1/*1*	Normal	**1/*1*	2	Normal
PSY 6	**1/*1*	Normal	**1/*1*	2	Normal
PSY 7	**1/*1*	Normal	**1/*10*	1.25	Normal
PSY 10	**1/*1*	Normal	**1/*10*	1.25	Normal
PSY 13	**1/*1*	Normal	**1/*10*	1.25	Normal
PSY 19	**1/*1*	Normal	**1/*41*	1.5	Normal
PSY 21	**1/*1*	Normal	**1/*10*	1.25	Normal

All patients, recruited from the psychiatric setting, with a genotype-inferred normal metaboliser status, except PSY 13, were being co-administered inhibitors of CYP2C19 and/or CYP2D6. PSY 13 had measured concentrations of amitriptyline and nortriptyline within the expected ranges. The rest of the 'normal metabolisers' – PSY 5, 6, 7, 10, 19 and 21 – had amitriptyline + nortriptyline concentrations above the expected range. PSY 13 presented the highest hydoxy metabolite to parent concentration ratios among the 'normal metabolisers'. Consideration of concomitant drugs allows further interpretation of the concentration ratios:

PSY	[Z- + E-10-OH AMI] to AMI ratio	[Z- + E-10-OH NOR] to NOR ratio	*Concomitant Inhibitor Risk*		NOR to AMI ratio
			CYP2D6	CYP2C19	
13	0.33	3.04	None	None	0.4
7	0.19	2.04	None	Weak/Moderate	0.2
10	0.17	1.56	None	Weak/Moderate	0.2
21	0.10	0.94	Weak/Moderate	None	0.9
6	0.04	0.44	High	None	0.8
19	0.03	0.38	High	None	0.3
5	0.06	0.30	High	Weak/Moderate	0.8

The hydroxy-metabolite to parent concentration ratios were highest in patients with no risk of CYP2D6 inhibition and lowest in patients at high risk of CYP2D6 inhibition. The nortriptyline to amitriptyline ratio was lower in the patients with weak-moderate risk of CYP2C19 inhibition, compared to those at no risk, with the exception of PSY 5. The latter, however, had high risk of CYP2D6 inhibition which may result in higher nortriptyline levels, and thus, a higher nortriptyline to amitriptyline ratio.

PSY 1	**1/*1*	Normal	**2A/*4*	1	Intermediate
PSY 12	**1/*1*	Normal	**4/*10*	0.25	Intermediate
PSY 14	**1/*1*	Normal	**10/*10*	0.5	Intermediate
PSY 15	**1/*1*	Normal	**10/*10*	0.5	Intermediate
PSY 20	**1/*1*	Normal	**4/*41*	0.5	Intermediate

Annotation For CYP2C19: Normal metabolism of amitriptyline.
For CYP2D6: Reduced metabolism to less active compounds, compared to normal metabolisers. Higher blood concentrations of active drug may increase the probability of side effects.
Consider 25% reduction of recommended starting dose.

The Cmin concentration of amitriptyline + nortriptyline, normalised to the dosing schedule, was highest in PSY 1 and PSY 20, while the ratios of hydroxy-metabolites to parent were lowest in these two patients, compared to PSY 12, 14 and 15. Patients PSY 1 and PSY 20 were being co-administered CYP2D6 inhibitors denoting high risk of inhibition. The dose-reduction recommendation transpires most pertinent to PSY 1 and PSY 20.

Patient	CYP2C19		CYP2D6		
	Genotype	Metaboliser Status	Genotype	Activity Score	Metaboliser Status *updated post-March 2019*
PSY 9	**1/*2*	Intermediate	**2A/*4*	1	Intermediate
Annotation	For CYP2C19: Reduced metabolism of amitriptyline, compared to normal metabolisers. For CYP2D6: Reduced metabolism to less active compounds, compared to normal metabolisers. Higher blood concentrations of active drug may increase the probability of side effects. Consider 25% reduction of recommended starting dose.				

Patient PSY 9 had amitriptyline and nortriptyline concentrations above the expected ranges, and a concerning amitriptyline + nortriptyline concentration of nearly 390 ng/mL, as measured 14.5 hours post 75mg once daily dose. Patient PSY 9 was also being co-administered CYP2D6 inhibitors denoting high risk of CYP2D6 inhibition. The ratios of hydroxy-metabolites to parent were on the lower end of the range observed in the cohort. The dose-reduction recommendation becomes particularly important in this case.

PSY 17	**1/*2*	Intermediate	**1/*4/xN*	1-2	Intermediate/Normal
Annotation	For CYP2C19: Reduced metabolism of amitriptyline, compared to normal metabolisers. For CYP2D6: Reduced/Normal metabolism to less active compounds. Initiate therapy with recommended starting dose or consider 25% reduction of recommended starting dose.				

PSY 17 had amitriptyline levels above the expected range, nortriptyline levels below the expected range, and a low nortriptyline to amitriptyline concentration ratio (0.1), corresponding to the CYP2C19 intermediate metaboliser status. The CYP2D6 genotype of patient PSY 17 represents 2 possibilities: **1x2/*4* (normal metaboliser) or **1/*4x2* (intermediate metaboliser). The ratio of hydroxy-metabolites to parent concentrations approximates the values observed in normal CYP2D6 metabolisers. This subject would however warrant further investigation.

PSY 3	**1/*17*	Rapid	**1/*4*	1	Intermediate
PSY 11	**1/*17*	Rapid	**1/*4*	1	Intermediate
PSY 18	**1/*17*	Rapid	**1/*4*	1	Intermediate
Annotation	For CYP2C19: Increased metabolism of amitriptyline, compared to normal metabolisers, which may affect response or side effects. For CYP2D6: Reduced metabolism to less active compounds, compared to normal metabolisers. Higher blood concentrations of active drug may increase the probability of side effects. Consider alternative drug not metabolised by CYP2C19. If amitriptyline is warranted, utilize therapeutic drug monitoring to guide dose adjustment.				

The ratios of nortriptyline to amitriptyline concentrations for patients PSY3, 11 and 18 were on the highest end of the range observed in the cohort (1.5-2.0). An 8-fold variation in the normalised amitriptyline + nortriptyline concentration was observed among these subjects, reported to have an identical metaboliser status. PSY 3 and PSY 11 were at weak-moderate risk of CYP2D6 and CYP2C19 inhibition respectively. PSY 18, who was at high risk of CYP2D6 inhibition by concomitant drugs, had the highest normalised amitriptyline + nortriptyline concentration and the lowest hydroxy-metabolites to parent concentration ratios between the three patients. Therapeutic drug monitoring may indeed be useful in such cases.

3.3.4 CYP450 enzymes, serum concentrations and side-effects

Preliminary inferences on side-effect measures were gathered from an evaluation, conducted during data collection, on twenty-six (26) patients, 5 males and 21 females, categorized in two groups, with comparable age (range: 24–79 years): thirteen (13) patients, being followed by a consultant psychiatrist, who had been receiving 25–75 mg amitriptyline daily for over 12 months; and thirteen (13) patients, being followed by a consultant anaesthetist, who had been receiving 10 mg amitriptyline daily for less than 12 months.

Irrespective of the group – dose, duration of use, and indication – patients reported, on average, three (3) side-effects on ASEC which they associated to amitriptyline (median 3, range 0–8, in pain patients on 10 mg daily for less than 12 months; median 2, range 0–10, in psychiatry patients on 25–75 mg daily for over 12 months). Out of the 21 symptoms in ASEC, insomnia and decreased appetite (listed as uncommon and rare undesirable effects in the EU-SmPC, respectively) were the only two not reported by any of the subjects.

The most reported side-effect overall was drowsiness (14 out of 26, 54%), with subsequent frequencies as follows: Drowsiness (54%) > Dry mouth (35%) > Blurred vision (19%) = Feeling like the room is spinning (19%) = Tremor (19%) > Constipation (15%) = Palpitations (15%) = Feeling light-headed on standing (15%) > Headache (12%) = Diarrhoea (12%) = Increased appetite (12%) = Problems with sexual function (12%) = Weight gain (12%) > Disorientation (8%) = Yawning (8%) > Nausea or vomiting (4%) = Problems with urination (4%) = Sweating (4%) = Increased body temperature (4%).

Drowsiness was reported more frequently in pain patients on 10 mg daily for less than 12 months (11 out of 13, 85%) compared to psychiatry patients on 25–75 mg daily for over

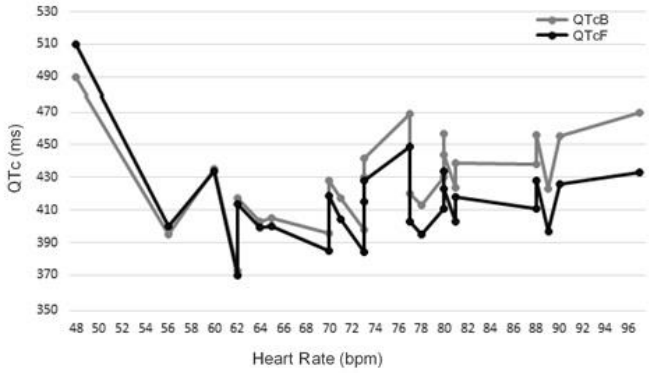
12 months (3 out of 13, 23%). Fisher's exact test two-tailed P value of 0.0048 suggested that the latter observation is statistically significant. The frequency of drowsiness contrasts with dry mouth, which tended to be reported more by psychiatry patients receiving higher amitriptyline doses for a longer time-frame compared to pain patients on lower doses over a shorter course (6 out of 13, 46%; 3 out of 13, 23%; respectively). With respect to intensity, the highest reported score for dry mouth was 3 (severe), observed in 6 psychiatry patients and 1 pain patient, whereas for drowsiness the highest score reported was 2 (moderate), observed in 2 psychiatry patients and 9 pain patients. Drowsiness and dry mouth were identified as the specific side-effects to study in subsequent analyses.

Patients underwent an electrocardiographic (ECG) examination, and each 12-lead ECG report was analyzed for heart rate and QT corrected by the Bazett's and Fridericia's formulae. Figure 3-22[A] portrays QTc, as corrected by the Bazett's formula (QTcB) and Fridericia's formula (QTcF) for the 26 patients, highlighting the differences between QTcB and QTcF as heart rate increases. Figure 3-22[B] depicts a significant correlation between the percentage difference QTcB–QTcF and heart rate ($P < 0.01$; Pearson correlation 1-tailed test).

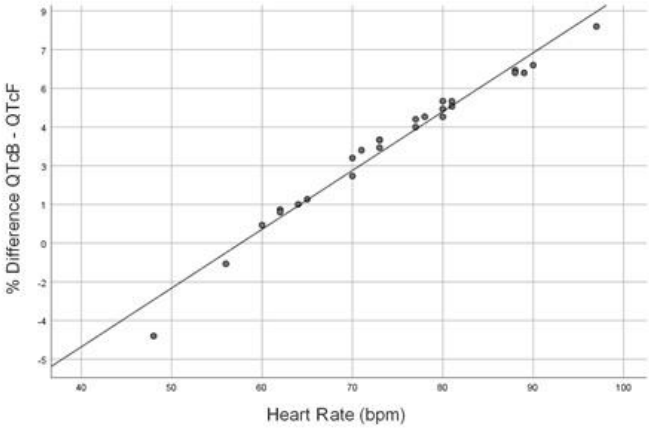
The data indicated that Bazett's correction formula potentially underestimates QTc at heart rates below 60 bpm and overestimates QTc at elevated heart rates. Considering QTcB, 6 subjects would be considered to have their QT prolonged (>450 ms, 2 in the 10 mg group and 4 in the higher-dose group) while considering QTcF only one patient (in the higher-dose group) is considered to have QT prolongation. Fridericia's correction may be more appropriate in subjects with altered heart rates, and was the preferred correction for subsequent analyses.

Figure 3-22: Preliminary assessment of ECG measures – QT correction

[A] n=26
QT corrected with Bazett's formula (QTcB) and Fridericia's formula (QTcF) for each patient *vs* Heart Rate



[B] n=26
Percentage difference between QTcB and QTcF *vs* Heart Rate



Outcomes of the preliminary assessment are detailed in the published article[61]. Upon complete recruitment (total of 44 participants), the ECG data for the entire cohort was reviewed to identify potential: (1) PR interval prolongation, (2) widening of the QRS-complex, and (3) QT interval prolongation. In the assessment of the electrocardiographic reports, the PR interval was considered prolonged if >200 ms (Aro et al, 2014), the QRS-complex was considered widened if >120 ms (Gupta & Thakur, 2001), and the QTcF interval was considered prolonged if >450 ms (ICH-E14, 2005). Out of the 44-participant

[61] Mifsud Buhagiar L, et al. Safety implications of low-dose amitriptyline in neuropathic pain. *Pharm Front* 2019;1:e190003. doi:10.20900/pf20190003.

cohort, 2 patients had QTcF prolongation, while PR prolongation and QRS widening were identified in 2 and 4 patients respectively. These 8 patients were considered to have an abnormal ECG.

An abnormal ECG result was observed to be more likely in patients at high risk of CYP2D6 inhibition by concomitant drugs – $X^2(2)=7.152$, P=0.028. Patients with abnormal ECGs had significantly higher mean nortriptyline concentrations at time of blood withdrawal (Ct, 61.68 ng/mL), compared to patients whose ECG did not present QTcF prolongation, QRS widening or PR prolongation (Ct, 31.88 ng/mL). This correlation (P=0.028) was upheld when nortriptyline Cmin levels, normalised to the dosing schedule (n=33), were tested. Inclusion of normalised Cmin nortriptyline concentrations as covariate and CYP2D6 inhibition risk as factor, in a logistic regression model with stepwise forward entry, identified the risk of CYP2D6 by concomitant drug(s) as the lone predictor in the parsimonious model for abnormal/normal electrocardiogram, explaining 28.3% variation in the ECG outcome (P=0.036).

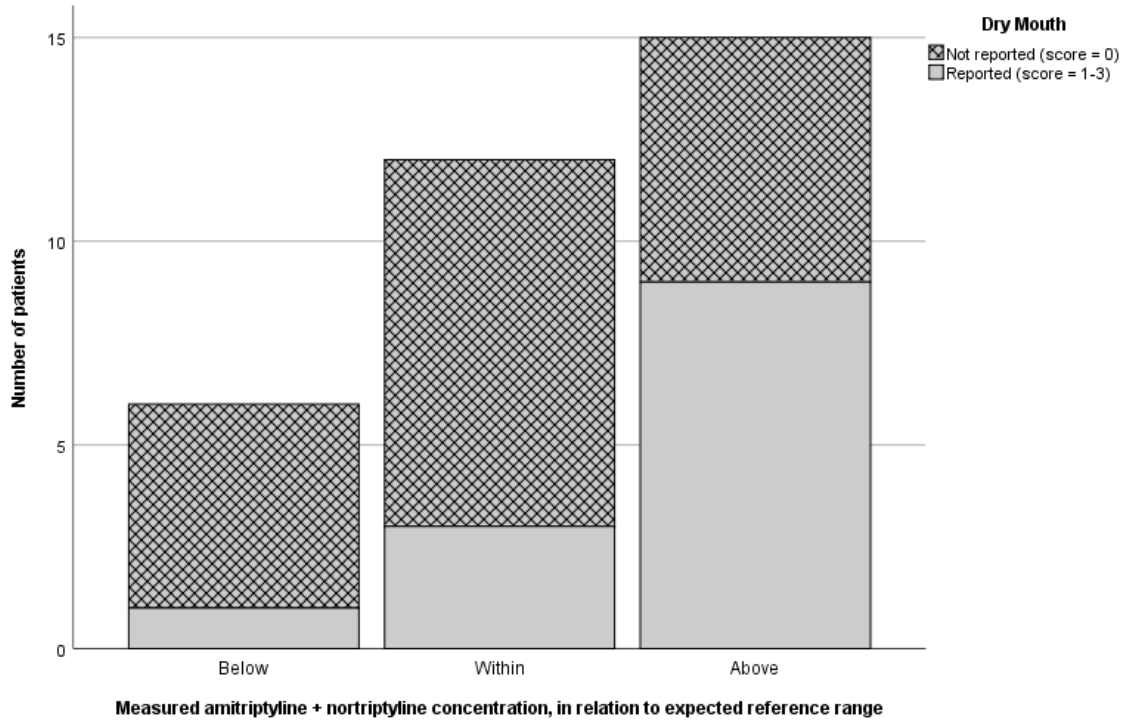
CYP2C19 and CYP2D6 genotypic measures and risk of CYP inhibition by concomitant drugs, did not render significant correlations to total side-effect burden and neither to the dry mouth or drowsiness scores (n=42). Total side-effect burden was not correlated to any of the measured concentrations. As for the two specific side-effects under study, dry mouth score was positively correlated to the concentrations of amitriptyline, nortriptyline, amitriptyline + nortriptyline, and Z-10-OH nortriptyline as measured at time of blood withdrawal (R=0.351, P=0.020; R=0.316, P=0.037; R=0.360, P=0.016; R=0.355, P=0.018; respectively). The total concentration of hydroxy-metabolites (Z- and E-10-OH NOR + Z- and E-10-OH AMI) was on average 34.29 ng/mL in patients reporting dry mouth, compared to 28.55 ng/mL who scored zero for dry mouth. The positive

correlations between dry mouth score and amitriptyline, and amitriptyline + nortriptyline concentrations, were upheld when Cmin levels, normalised to the dosing schedule, were tested. It is noted that for patients whose amitriptyline + nortriptyline concentration was above the expected dose-related reference range, the mean dry mouth score reported is higher (Table 3-29, Figure 3-23, n=33).

Table 3-29: Dry mouth score according to amitriptyline + nortriptyline concentration

Amitriptyline + Nortriptyline concentration	Sample Size	Mean Dry mouth score	Std. Deviation	P-value	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Below expected range	6	0.17	0.408		0.00	0.60
Within expected range	12	0.67	1.231	0.044	0.00	1.45
Above expected range	15	1.73	1.486		0.91	2.56

Figure 3-23: Dry mouth reports according to measured amitriptyline + nortriptyline concentration in relation to the expected dose-related reference range



In contrast, drowsiness was negatively correlated to the measured concentrations at time of blood withdrawal (Ct), particularly nortriptyline, amitriptyline + nortriptyline, and Z-10-OH nortriptyline (R=-0.361, P=0.016; R=-0.304, P=0.045; R=-0.368, P=0.014; respectively). A negative relationship was apparent between drowsiness score and daily amitriptyline dose (Spearman correlation = –0.312, P = 0.039). This negative correlation between drowsiness score and nortriptyline concentration, was upheld when Cmin levels, normalised to the dosing schedule (n=33), were tested.

A time parameter was added in investigating these outcomes, categorising patients into: having been administered amitriptyline for (i) less than 12 months, or (ii) over 12 months. The incidence of drowsiness was higher in those that had been on amitriptyline for less than 12 months; $X^2(3)=12.522$, P=0.006. The drowsiness severity score was also higher in those that had been on amitriptyline for less than 12 months (P=0.002). This was unanticipated since daily dose in patients who had been on amitriptyline for less than 12 months was significantly lower (P=0.000). Drowsiness incidence was not significantly related to the dose but severity score was negatively correlated to the dose. The higher the dose, and corresponding concentrations, the lower the drowsiness score. This may imply that over time, even upon dose escalation, drowsiness is perceived less problematic and a lower score is rated. This scenario was not replicated for dry mouth and total side-effect burden. The latter was unrelated to time. There was, however, a large percentage of patients who had been on amitriptyline therapy for over 12 months that reported dry mouth (53.1%) compared to patients with less than 12 months of therapy with amitriptyline (16.7%). The percentage difference (36.4%) is significant because the p-value (0.030) is less than the 0.05 criterion. The severity score of dry mouth was higher in patients being on amitriptyline for more than 12 months (higher doses and concentrations); P=0.017.

Logistic regression modeling for dry mouth and drowsiness (as dichotomous variables: reported or not reported), with amitriptyline + nortriptyline measured concentration and dose included as factors, and time included as covariate, yielded parsimonious models with time since onset of therapy with amitriptyline as the lone significant predictor of side-effect incidence. Considering the model for drowsiness, the odds ratio indicates that patients having been on amitriptyline therapy for less than 12 months are 11 times more likely to report drowsiness compared to their counterparts with over 12 months therapy (R^2=0.274, P=0.002, n=44). As for the dry mouth model, the odds ratio indicates that patients with having been on amitriptyline therapy for more than 12 months are 6 times more likely to experience dry mouth compared to patients with less than 12 months therapy (R^2=0.148, P=0.024, n=44).

Repeating the logistic regression modelling exercise for the 33 patients with Cmin levels normalised to the dosing schedule, allowed further interpretation. In the model for dry mouth, including time as factor, and Cmin concentrations for amitriptyline, and amitriptyline + nortriptyline as covariates, did not give significant results. The association between incidence of dry mouth and time since amitriptyline therapy onset, is faded. In the model for drowsiness, including time as factor, and Cmin nortriptyline concentration as covariate, generated a parsimonious model with time since onset with amitriptyline therapy as lone predictor explaining 38% of the variation in drowsiness incidence. Patients who have been on amitriptyline therapy for less than 12 months are around 15 times more likely to report drowsiness than those with over 12 months therapy (P=0.001, n=33). An element of tolerance to the sedative effects of amitriptyline over time may explain these observations.

As a final point, dry eye was reported by 5 out of the 24 patients recruited from the POP Clinic who were explicitly questioned about this effect, with a score of 1 (4 patients) or 3 (1 patient). The dry eye effect was not investigated further, although it appeared to be unrelated to the daily dose of amitriptyline.

3.4 Abridgement

Regulatory inferences (Results; section 3.1) draw attention to the official FDA labelling for amitriptyline, which distinguishes CYP2D6 poor metabolisers but does not quantify the recommended dose adjustments and makes no direct reference to CYP2C19. The harmonised EU-SmPC, as per the CHMP opinion and Commission implementing decision of 2017, includes pharmacogenetic considerations related to CYP2C19 or CYP2D6 poor metabolisers, with corresponding recommendation for dose alterations. This information, implementable in the product information via variations at national level, was not included in the SmPCs of all amitriptyline products at time of evaluation, with discrepancies being evident even within the same Member State. The limited reference to implications of genetically determined CYP activity and the potential of phenoconversion was observed through causality assessment of drug interaction reports, involving amitriptyline and CYP2D6 inhibitors/substrates listed in the amitriptyline SmPC. Interpretation of the drug interaction reports, as well as dry mouth and sedation Individual Case Study Reports (ICSRs) in relation to amitriptyline, was often complicated in view of fragmented submissions, unclear descriptions of the sequence of events and the absence of details on the administered dose or drug blood level measurements.

Analytical developments (Results; section 3.2) in establishing the means of measuring serum concentrations, progressed from trialing with high performance liquid chromatography (HPLC), to ultra-high performance liquid chromatography (UHPLC), and more successfully with a new LC-MS/MS validated method which allows simultaneous quantification of amitriptyline, nortriptyline and their hydroxy-metabolites in less than six minutes. Experimentation with kits made available for genotyping *CYP2D6* and *CYP2C19* enabled substantial exposure to the complexity of the scenario, particularly when working with alternative biological sources to whole blood, such as buccal swabs. The latter rendered effective sources for extracting DNA intended for real-time PCR with TaqMan® SNP Genotyping. The results and their interpretation provided functional data on the recruited patients.

Clinical observations (Results; section 3.3) were primarily construed on the lab-reported genotype/phenotype results for CYP2C19 and CYP2D6. Patients identified to be at risk of CYP2C19 or CYP2D6 inhibition by concomitant drug(s), were generally phenotyped as normal metabolisers in the genotyping report. Updating the CYP2D6 metaboliser status in line with the 2019 CPIC Consensus distinguishes a higher proportion of patients as intermediate CYP2D6 metabolisers. Nonetheless, the potential of phenoconversion persisted as an important parameter in the analyses.

All measured serum concentrations – amitriptyline, nortriptyline, and their hydroxy metabolites – were positively correlated to the daily dose of amitriptyline administered in the respective patient. The nortriptyline to amitriptyline concentration ratio was unrelated to the daily amitriptyline dose. CYP2C19 metaboliser status represented the significant main effect in relatively explaining the variation, with intermediate metabolisers having lower mean nortriptyline to amitriptyline concentration ratios, and rapid metabolisers

having higher mean ratios, compared to normal CYP2C19 metabolisers. The mean ratio of hydroxy-metabolites to parent was lower in patients at high risk of CYP2D6 inhibition by concomitant drugs, compared to patients for whom the risk was conceivably inferior, and was significantly positively correlated to the CYP2D6 activity score estimated in line with the 2019 update. Comprehensive data analysis, including multiple computational measures, enabled further investigation. The amitriptyline + nortriptyline concentrations of most patients were outside the expected dose-related reference ranges. The likelihood of amitriptyline + nortriptyline concentrations being above the expected ranges was observed to increase with increased risk of CYP2D6 inhibition by concomitant drugs. The intricate observations were additionally rationalized by revising the CYP2D6 metaboliser status in line with the risk of CYP2D6 inhibition by concomitant drug(s), rather than considering genotyping results alone.

Genotype-inferred CYP2D6 and CYP2C19 metaboliser status did not render significant correlations to dry mouth or drowsiness scores, and neither to the total side-effect burden. An abnormal ECG result was observed to be more likely in patients with high risk of CYP2D6 inhibition by concomitant drug(s), having higher mean nortriptyline concentrations. The preliminary inferences that total side-effect burden was unrelated to the amitriptyline dose and duration of use were later confirmed whereby the number of patient-reported side-effects yielded no significant relationship to any of the scrutinized measures. While positive correlations were observed between dry mouth and serum concentrations under study, negative correlations were obtained for drowsiness. The initial observation that drowsiness was most evident in patients on less than 12 months amitriptyline therapy was supported by the results of the complete cohort when the time-parameter was included in the analysis.

Chapter 4
Discussion

4.1 Synthesis of the literature and principal findings

The discussion that unfolds in this section is centred on the principal findings of the research conducted and critical engagement with published work in the field. Pharmacogenetics is prominent in the literature vis-à-vis a patient-centred approach to medicine. While conventional medicines are generally developed for an unselected population, personalised medicine encompasses stratified medicine in which patients are selected on the basis of biomarkers for efficacy and safety, and individualised medicine whereby therapy is actively personalised for the patient. The degree of precision and accuracy of pharmacotherapy is expected to increase by mitigating the possibility of missing an intended benefit because of blood levels that may be too low, and the risk of adverse effects related to elevated drug concentrations. The risk-benefit principle shapes the basis of the regulatory framework. In turn, the systems adopted by regulators may support, or otherwise, the translation of pharmacogenetic data into recommendations implementable in clinical practice. Taking amitriptyline as a case example, the first research question was: *Is the regulatory infrastructure supporting the implementation of precision pharmacotherapy by integrating pharmacogenetics in the product information and throughout the evaluation of safety concerns?*

Enhanced knowledge and technological advances allowing efficient characterization of relevant genetic variants in clinical trial participants have unfolded new opportunities to investigate pharmacogenetics during the development of novel medicines. In this regard, regulatory agencies globally have been developing guidance for drug developers, providing a framework for exploiting pharmacogenetic data to optimize drug pharmacokinetic parameters (Maliepaard et al, 2013). The Food and Drug Administration (FDA) has conducted a series of workshops in collaboration with the Drug

Information Association to inform drug developers, support policy development and enhance implementation of pharmacogenetics. The European Medicines Agency (EMA) is implementing a policy of transparency and involvement of stakeholders, for instance in scientific advice and in collaborations with the Pharmacogenomics Working Party, as exemplified by open conferences that disseminate information to ensure balanced understanding of the contribution of validated pharmacogenetic tests to public health.

The significance of genomic data in evaluating drug safety and efficacy progressed in all phases of drug development, from early clinical trials to post-marketing assessment, with genomic information relevant to benefit/risk evaluation being increasingly incorporated in drug literature. To accumulate such data, genomic sample collection is encouraged by all ICH regulatory agencies as the collection rate is minimal in many regions (ICH Final Business Plan E18, 2015). Guided by the magnitude of the pharmacogenetic effect, the robustness of available evidence and the overall benefit-risk balance, inclusion of genomic information in product labelling may influence clinical use and pharmacovigilance activities.

Labels may carry mandatory information for the prescriber to adopt, important recommendations for the use of the drug, or data for information purposes only. The number of drugs with CYP genetic information in their product information is steadily increasing (Reis-Pardal et al, 2017). Initial evidence may guide regulators to include reference to pharmacogenomics in those label sections which are mainly only intended to provide information. Thereafter, based on further evidence gathered post-authorisation, possible pharmacogenomic implications are reallocated towards sections within the hierarchy of information relevant to clinical decision making, like Therapeutic indications, Posology and Contraindications (Ehmann et al, 2015).

The FDA has, since 2008, issued a list of genomic biomarkers in the context of approved drug labelling[62]. *CYP2D6* is the listed biomarker for amitriptyline with corresponding precautions included in the labelling text. He et al (2011) highlight the existing gap between the knowledge of drug-related copy number variations and implementation of drug label changes. Conrado et al (2013) report that the genetic differences in drug metabolism are recognised in FDA-approved drug labelling, particularly when clinically relevant interactions trigger dose adjustments or alternative drug use. In comparing the cytochrome P450 pharmacogenetic information included in US FDA drug labels and EU Summary of Product Characteristics (SmPCs), Reis-Pardal et al (2017) note that centralized SmPCs were found to have higher quality scores than decentralized SmPCs. Quality assessment was based on accessibility, reliability, completeness and applicability of the information. The authors conclude that, irrespective of the time since last review, a higher overall quality score was pegged to US labels compared to the EU SmPCs.

The case of amitriptyline, represents a promising example in this context, whereby the 2017 regulatory developments in the EU towards a harmonised SmPC, include pharmacogenetic considerations with respect to CYP2D6 and CYP2C19. Undoubtedly, a major step forward with respect to actionable pharmacogenetic data in the product information of an established drug. The changes to the amitriptyline SmPC, however, are implementable at national level. Through the investigation carried out within this research, considering the UK, just about 60% of accessible amitriptyline SmPCs were confirmed as updated in line with the relevant text upon inspection (Results; section 3.1.1). A number of reasons may prolong implementation of variations at national level. Marketing Authorisation Holders (MAHs) may take longer than expected to file

[62] Food and Drug Administration. Table of Pharmacogenomic Biomarkers in Drug Labeling [Online]. US-FDA [accessed 2018 Aug 11]. Available from: https://www.fda.gov/downloads/Drugs/ScienceResearch/UCM578588.pdf.

variations for their products, particularly for amendments deemed less critical, possibly grouping all changes in a planned subsequent submission. A number of national competent authorities have a considerable backlog in validating and processing such submissions, inundated by issues of high risk and urgency, which warrant priority. Irrespective of the numerous explanations and justifications, which may be logical and pragmatic, harmonisation is far from being accomplished. Healthcare professionals, assuming that they do access SmPCs, are being presented with variable information within the same Member State, and possibly more so if one were to extend such an exercise by assessing the SmPCs in other EU countries and beyond.

These observations lead to a recommendation for centralized initiatives to mitigate divergences. MAHs are required to submit information to the Article 57 database, published by the EMA as of July 2018, in accordance with Article 57(2) of Regulation (EC) No. 726/2004[63], which shall include product name, active substance, route of administration, country of authorisation, country of location of the pharmacovigilance system master file, and MAH details on all medicinal products authorised in the European Economic Area. Inclusion of SmPCs and patient leaflets for all authorised medicines, including nationally approved ones, could represent a significant step towards harmonisation of the information available for medicinal products and could further enhance safety and vigilance.

Summary of Product Characteristics (SmPCs) for antidepressants promote safe use by providing instructions for clinical monitoring, particularly of non-somatic symptoms, with disputed applicability in clinical practice (Nederlof et al, 2015). Being an integral

[63] Regulation (EC) No 726/2004 of the European Parliament and of the Council of 31 March 2004 laying down Community procedures for the authorisation and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency Official Journal L 136 , 30/04/2004 P. 0001 – 0033.

element of the marketing authorization process, SmPCs have strictly defined structure and content, which should be consistently updated over the life cycle of the product (Reis-Pardal et al, 2017). The example of amitriptyline substantiates the realization that safety information of a medicinal product is not complete at the time of authorization, the diversity in patient populations may not be entirely foreseen and a number of limitations in the data are addressed after the product enters the market. Data collection, reporting and evaluation in the post-authorisation setting has absorbed part of the preauthorisation burden in reassuring that the benefit–risk ratio remains favourable (Borg et al, 2015).

Post-approval safety monitoring of medicines is a global endeavour, underscored by EU and US legislation remarkably in the last decade. Although the details of pharmacovigilance requirements placed on industry and regulators may differ among regions, the common approach is based on increased transparency, accountability and robust science and activities for post-marketing serviellance, which supports collaboration. Global pharmacovigilance alliance is embodied in the World Health Organisation (WHO) and the Uppsala Monitoring Centre as vital players in a mutual struggle to reduce the harm that may be caused by medicines. ICSRs (individual case safety reports) from patients and healthcare providers in member countries of the WHO Programme for International Drug Monitoring are central sources of data. The WHO lists (i) a national pharmacovigilance centre, (ii) a national spontaneous reporting system, (iii) a national database for collating and managing ADR reports, (iv) a national advisory committee and (v) a clear communication strategy, as minimum requirements for a functional national pharmacovigilance system[64].

[64] World Health Organisation. Minimum Requirements for a functional Pharmacovigilance System [Online]. WHO; Geneva: 2010 [accessed 2019 Jul 28]. Available from: http://www.who.int/medicines/areas/quality_safety/safety_efficacy/PV_Minimum_Requirements_2010_2.pdf.

European directives and regulations introduced between late 2010 and 2012 enable the collection of adverse drug reaction information from all available resources to increase the information pool for the analysis of relevant signals (Borg et al, 2015). Data collection through spontaneous reports presents issues since treatment allocation is not randomised, medical history and data on concomitant medications is often unavailable, and an event caused by an interaction may be reported as a disparate reaction. The scenario is further complicated by the existence of duplicate reports submitted from diverse sources or case follow-up being considered as new reports, set reporting requirements which may increase proportion of serious reactions reported compared to non-serious ones, and reporting patterns which may change over time, influenced by marketing and media attention (Bate & Evans, 2009).

In assessing suspected cases of drug interaction in the EU EudraVigilance Data Analysis System, in relation to amitriptyline as example of an established drug with actionable pharmacogenetic recommendations, the limited number of follow-up reports was evident in this research. One possible explanation is that reporters may await until all information is available before they submit their report. Considering the lack of complete information in the cases, particularly with respect to investigations, this supposition is quite implausible. Submission of a follow-up report, including data which was not available at first instance, could further assist in the assessment of the case. This is discussed in a number of the reports assessed - a reporter explains how a case was changed from 'non-serious' to 'serious' upon review of follow-up data, highlighting their importance, while few others claim a follow-up was requested but not submitted or the patient refused to provide further details (Results; section 3.1.2). Reporters may not perceive the value of follow-up reports while Marketing Authorisation Holders may not have interest in chasing reporters for additional data on a case they shelved as 'unassessable'. These

observations lead to a recommendation for strengthening the role of regulators to act at the forefront in enhancing quality of adverse drug reaction reports and their assessment by putting forward, for instance, a standard procedure for sending a list of applicable questions to the reporters, as an example of good practice in this area of pharmacovigilance.

In the real world, the authorised drug is presented to categories of patients which may have been under-represented in clinical trials, who may endure prolonged exposure, with conditions being no longer carefully monitored. Post-authorisation activities for genomic data collection and safety signal detection are recommended in the EMA 2015 Guideline on key aspects for the use of pharmacogenomics in the pharmacovigilance of medicinal products[65]. The guidance makes reference to *CYP2D6* testing implications for increased surveillance, alternative dosing or drug avoidance to prevent increased exposure to drug or metabolite and ADRs arising through genomic biomarkers linked to pharmacokinetics. Genomic information may be generated using data from non-clinical studies, clinical studies, epidemiological studies, and well-documented ADR case reports which can generate valuable information on the relationship between the genotype or phenotype and the clinical feature of adverse reactions. Measurement of drug concentrations in patients who experience serious ADRs can provide clinically relevant data which may help optimise therapy. Idiosyncratic reactions related to individual genomic traits and the occurrence of therapy failure as a result of pharmacogenomic influence are to be investigated in the post-authorisation period and any signals or emerging safety issues reported to regulatory agencies. Risk minimisation measures may involve laboratory investigations such as genotyping and therapeutic drug monitoring.

[65] European Medicines Agency. Guideline on key aspects for the use of pharmacogenomics in the pharmacovigilance of medicinal products [Online]. CHMP; EMA: 2015 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2015/11/WC500196800.pdf.

This primes the discussion on the analytical part of the research. The investigation of blood levels to look into parent drug, metabolites and concentration ratios, which could provide relevant pharmacokinetic data, may not be comprehensively included in the portfolio of many laboratories. Furthermore, genotyping for highly polymorphic genes may entail resource intensive analytics. Taking amitriptyline as a case example, the second research question was: *What are the analytical and technical requisites for the application of a pharmacogenetic approach to guide decisions in practice?*

Amitriptyline endures as a pharmacotherapeutic choice for the management of depression, decades after its first authorization and use in clinical practice (Barbui & Hotopf, 2001). The latter is driven by amitriptyline's efficacy profile (Cipriani et al, 2018), whereas the narrow therapeutic index and side effect concerns warrant the recommendations for therapeutic drug monitoring (Hiemke et al, 2018). Back in 1984, Dawling and colleagues, through an amitriptyline dose-prediction test based on plasma concentrations after a single dose, highlighted that patients, particularly the elderly, may benefit in having an adequate, safe dose calculated on the blood levels, rather than opting for the routinely prescribed dose (Dawling et al, 1984).

In amitriptyline psychiatric therapy, combined amitriptyline and nortriptyline serum concentrations between 120 and 250 ng/mL are anticipated. Additional response is unlikely above 450 ng/mL, whereas with serum concentrations around five times those required for antidepressant efficacy, cardiac conduction disturbances, seizures and coma are more likely. Reponse rates to tricyclic antidepressants are reported to increase from 30% to as high as 80% by the use of serum concentration monitoring (MacKichan & McGory, 2009). The latter is mainly employed when there is suspicion of noncompliance, inadequate response, toxicity or unsual pharmacokinetics.

As of 2017, the treatment of neuropathic pain in adults is included as a therapeutic indication for amitriptyline in the harmonised EU Summary of Product Characteristics, in which case an initial daily dose of 10 mg is recommended. The dosing rationale recognizes that pain relief may be achieved using lower doses than prescribed for depressive disorders and is intended to mitigate adverse events by gradually titrating to the lowest effective dose[66]. Monitoring blood levels of amitriptyline and its metabolites following administration of doses at the lower end of the dosing continuum, may prove challenging in laboratory practice.

This research strived to develop a practical method for routine analysis of amitriptyline and its metabolites in serum. Commencing with high-performance liquid chromatography (HPLC), a relatively simple approach was adopted to investigate the combined effect of pH and acetonitrile composition on performing efficient separation of similar tricyclic compounds, without resorting to complex additives and resource-intensive settings. In the process of HPLC system configuration and optimisation of the chromatographic conditions, methodical consideration was given to the properties of the stationary phase. Significant interaction between the compounds and HPLC column packing materials are common sources of peak tailing, asymmetry and low separation efficiencies (Ashour & Kattan, 2012). The sodium or potassium salts of phosphoric acid are commonly used in buffer systems for reversed phase HPLC. In general, no more than 50% organic solvent should be used in the mobile phase, depending on the concentration of the specific buffer employed. Methanol and acetonitrile are ordinarily employed as organic modifiers, with the latter preferred in this research due to lower UV absorbance at the wavelength used for analysing the compounds of interest (Rao & Goyal, 2016).

[66] EMA - European Medicines Agency. Assessment report Referral under Article 30 of Directive 2001/83/EC, CHMP [Online]. EMA; 2017 [accessed 2019 Jul 19]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Saroten_30/WC500227970.pdf.

In determining the amount of organic modifier, a content that is too low infers long retention times while opting for a high content leads to shorter retention times with decreased peak resolution (Vella et al, 2014). Separation and selectivity for ionisable compounds is affected by pH, primarily controlled through the buffering system in the mobile phase, which largely determines the concentration ratio of protonated and unprotonated forms in the aqueous mobile phase and their distribution between the non-polar stationary phase and the polar mobile phase. Additional considerations apply when observations from pH determined in aqueous systems are extrapolated to acetonitrile-aqueous systems. Unlike the buffer capacity, the pH of a buffer changes upon the addition of an organic solvent and so do the pKa values. The combined effect of these shifts may yield important differences under the measurement conditions (Neue et al, 2006, Subirats et al, 2007). Changes in pH may also alter the position and intensity of UV absorption bands of molecules. The working pH is preferably selected based on the type of buffer being used and according to the pKa values of the compounds. Alkaline pHs are not recommended for HPLC applications due to potential damage to the column through solubilisation of the silica support (Goeringer et al, 2003, Kirkland et al, 1995). Consequently, all mobile phases considered in the development of the method had pH values lower than the pKa value of the analytes.

Various parameters were modified to establish effective separation conditions, taking into account the physicochemical properties of the analytes. The rapid systematic technique presented scrutinises the combined effect of two major analytical parameters, buffer pH and mobile phase composition, which impact on the resolution of chromatographic peaks in the simultaneous separation of amitriptyline, nortriptyline, E- and Z-hydroxyamitriptyline, E- and Z- hydroxynortriptyline, and clomipramine as internal standard (Results; section 3.2.1). With due consideration to the pertinent sample

preparation procedures and ensuing validation, the proposed procedure is prospectively applicable to the analysis of pharmaceutical impurities. In effect, amitriptyline, is one of the known impurities in nortriptyline preparations (ElHoussini & Zawilla, 2014). As for pharmacokinetic studies, the low concentrations anticipated in patient serum samples necessitated further experimentation for an apposite method of analysis.

Linden and colleagues (2008) described a method for the determination of amitriptyline, nortriptyline, desmethylnortriptyline, E- and Z-10-hydroxyamitriptyline, and E- and Z-10-hydroxynortriptyline, in human plasma samples using HPLC coupled with a diode array detector. The lower limit of quantification (LLOQ) for all compounds evaluated was 5 ng/mL with complete separation achieved in around 22 minutes. Ultraviolet-visible detection is the predominant technique in HPLC for tricyclics, with mass spectrometry using different interfaces becoming preferred, as regards sensitivity and identification, for analysis in bio-samples (Uddin et al, 2011). The present research work describes a new, specific LC-MS/MS method for the simultaneous determination of amitriptyline, its active metabolite nortriptyline and their hydroxy-metabolites. The assay was validated in human serum, with a run time of 6 minutes and a lower limit of quantitation of 0.5 ng/mL, putting forward a practical method which may be adopted for therapeutic drug monitoring in amitriptyline therapy (Results; section 3.2.2). The developed method entails a simple extraction procedure, combined with a rapid and sensitive LC-MS/MS analysis. The validated method has been applied on real samples, demonstrating practical applicability in the field of clinical analysis.

Blood concentration ratios of nortriptyline to amitriptyline are reported to be lower in CYP2C19 poor metabolisers (Baumann et al, 1986, Shimoda et al, 2002), occurring in around 19% of Asians (Mizutani, 2003). The CYP2D6 enzyme is responsible for the

formation of the E-10 hydroxy-metabolites and poor metabolism, occurring in around 7% of Caucasians (Mizutani, 2003), or inhibition, may retard nortriptyline elimination and impair production of the major 10-hydroxynortriptiline enantiomer (Bertilsson et al, 2002, Breyer-Pfaff, 2004). To study the influence of CYP2C19 and CYP2D6 on blood levels, having the means of detecting single nucleotide polymorphisms (SNPs) in the genes of interest is essential. Being the most widespread type of polymorphisms identified in the human genome, SNPs are believed to be the main explanation for 90% of all types of genetic individual variations (Chaudhary et al, 2015) and detection technologies used to determine alleles of known polymorphisms in target sequences have evolved from labour-intensive, expensive processes, to higher efficiency methodologies that offer a degree of automation and are relatively lower in cost (Kwok & Chen, 2003). This thesis progressed in a most apposite time when the relevant equipment and technical requirements are becoming available in the standard armamentarium.

Biological sources that permit relative comfort for participants while enabling ease of access, storage, and transport, with contained costs are coveted for the application of DNA isolation and genetic testing in the clinical setting (Rethmeyer et al, 2013). Alternatives to whole blood, which entail less invasive collection, include buccal cell, hair with follicle, and urine. Buccal swabs are suitable for self-collection and are associated with reduced volumes of sample required, cost-effectiveness and durability. In view of the low demands of DNA required for genotyping, in the nanograms range, buccal swabs may provide sufficient quantities (Ghatack et al, 2013). Although blood samples were more practical during the initial experimentation with the TrimGen Mutector™ genotyping kits in this research, buccal swabs yielded effective sources for extracting DNA intended for real-time PCR with TaqMan® SNP Genotyping, whereby

only 1 out of 44 samples provided to X-Gene Diagnostics (US) for analysis failed due to low DNA concentration (Results; section 3.2.3).

Resorting to analysis in the US enabled the consideration of a higher number of alleles in the test panel. Parenthetically, in the 43 patients for whom a *CYP2C19* genotyping result was made available, the **9* allele, a decreased function allele with low frequency in the general population (0.00025 for Caucasians[67]), was identified in one patient. Analysis at X-Gene Diagnostics also supported determination of copy number variation (CNV), with particular relevance to *CYP2D6*. While most patients have two copies of the *CYP2D6* gene, a minority of patients may have multiple copies. Laboratories may indicate the result as 'duplication' which does quantify the number of allele copies present. Other labs, as was the case in this research, give an exact copy number (xN). Nonetheless, the result does not indicate which allele is duplicated. A CNV of 3 was indeed identified in 5 out of the 42 patients for whom *CYP2D6* genotyping results were made available. For 4 of these patients, the duplication rendered an ultra-rapid metaboliser status since involving normal function alleles. The **1/*4/xN* genotype reported for 1 patient, with a CNV of 3, triggered further considerations in view of **4* being a no function allele. Since the genotyping panel utilised has a relatively low coverage and no hybrid detection, this patient may be considered an ultra-rapid metaboliser if, for example, there are three **1* copies, and the **4* call is resulting from a 2D6/2D7 or 2D7/2D6 hybrid that is not picked up in the CNV determination - this scenario is yet unlikely. Pondman and colleagues (2018) comment that *CYP2D6*1/*4* often occurs in combination with gene multiplication; duplication of **1* leads to normal metabolism whereas no extra functional enzyme would result from multiplication of **4* which therefore leads to a predicted

[67] PharmGKB. Gene-specific Information Tables for CYP2C19 [Online]. PharmGKB [accessed 2019 Jul 19]. Available from: https://www.pharmgkb.org/page/cyp2c19RefMaterials.

intermediate metaboliser phenotype. Determination of the multiplied gene would simplify the accurate provision of phenotype prediction for such patients.

Gaedigk and colleagues (2017, 2018) discuss the inconsistency in *CYP2D6* classifications, with activity scores of 1 and 2.5 being highly contested on whether to group as genetic normal metabolisers, or intermediate and ultra-rapid metabolisers respectively. Further distinction of subjects with activity scores between 1 and 2 is also considered, with subjects having an activity score of 1 designated as slow genetic normal metabolisers and activity scores of 1.5 or 2 designated as fast genetic normal metabolisers (Gaedigk et al, 2017). Moreover, the crude classification of allele function overlooks substrate-dependent effects of *CYP2D6* allelic variants, which may be particularly relevant for *CYP2D6*10*. The assignment of a 0.5 activity score for the latter was also questioned by the authors, amplifying the review and discussions underway (Gaedigk et al, 2018). The lack of standardisation thwarts the interpretation of results and report comparisons. Incoherent groupings in the literature make the drawing of concordant recommendations difficult, particularly when the variants genotyped and grouping procedures are not clearly disclosed.

During the course of this research, in March 2019, the CYP2D6 experts involved in the Clinical Pharmacogenetics Implementation Consortium (CPIC) CYP2D6 Phenotype Standardization Project, reached consensus[68], with major changes implementable in the CPIC CYP2D6 genotype to phenotype table. Further to assigning an activity score of 1.0 as intermediate metaboliser; an activity score of 2.25 as normal metaboliser, and a 0.25 activity score to *CYP2D6*10*, the experts agreed to have a continuous scale for activity

[68] CPIC. CYP2D6 Genotype to Phenotype Standardization Project [Online]. CPIC; 2019 [accessed 2019 Jul 20]. Available from: https://cpicpgx.org/wp-content/uploads/2018/09/CYP2D6-Genotype-to-Phenotype-Standardization-Project_consensus.pdf.

score but, in view of limited data, decided against the addition of a rapid CYP2D6 metaboliser phenotype. In view that genotyping within the present research had been completed prior to the finalisation date of the consensus, the lab-reported outcomes were thenceforth reconsidered accordingly. Besides the assignment of an intermediate metaboliser status to more patients, the updated activity scores were used to assess their relationship to the ratio of hydroxy-metabolites to parent, yielding a significant positive correlation.

The assessment of *CYP2C19* represents a comparatively less complex scenario. Nonetheless, allele drop-out resulting in under-representation of an allele may ensue from preferential amplification of one of the heterozygous alleles; for instance, *CYP2C19 *2* not amplifying in **10/*10* patients, or the **10* assay not amplifying in **2/*2* patients. The phenomenon has also been reported for *CYP2D6*, with intra-patient validation by seperate methods of determining genetic variation being recommended (Scantaburlo et al, 2017).

Although the inconsistencies evident in the literature to date challenge interpretation, data points towards an increased frequency of phenotypic CYP2D6 poor metabolisers, compared to the frequency predicted by genotype (Llerena et al, 2014). Inter-individual variability observed in vivo within a genotype group is typically large, alluding to additional sources of variability. Gaedigk et al (2018) suggest that therapeutic drug monitoring data may be convenient for phenotype determination. Nonetheless, an absolute phenotype is difficult, if not impossible, to determine, with multiple individual factors involving competing/compensating metabolism pathways, herbal/drug-drug interactions, and physiological mechanisms such as inflammation affecting a patient's phenotype at a given time.

Informed by the effective developments, and cognisant of possible reservations and foreseeable challenges, on the regulatory and analytical side, the research proceeded to consider the clinical scenario, investigating the interplay between metaboliser status, blood levels and patient outcomes. Taking amitriptyline as a case example, the third research question was: *Does the assessment of dose-related reference ranges, metaboliser status and phenoconversion potential facilitate the interpretation of therapeutic drug monitoring and clinical outcomes alongside genotype-guided dosing recommendations?*

The potential of therapeutic drug monitoring for tailoring therapy with tricyclic antidepressants is evidenced in the literature although insufficiently conveyed through the official product information of well-studied compounds, such as amitriptyline (Hiemke et al, 2018). The authors of the latest guidance for therapeutic drug monitoring in neuropsychopharmacology expand on a practical sampling time for measuring blood levels, indicating that timing deviations when sampling closely before the next dose are less critical since at the end of the dosing interval the concentration-time curve flattens. Hiemke and associates (2018) highlight that the expected trough concentration should then be computed. The proposed calculations were considered and discussed with same authors, for adaptation to this research which studied a distinct cohort of patients on amitriptyline therapy (Results; section 3.3.1). Measured serum concentrations of amitriptyline, nortriptyline, and their hydroxy-metabolites were positively correlated to the daily dose of amitriptyline administered in the respective patient, as expected (Shimoda et al, 1997). The observation of E-10 hydroxynortriptyline being the most predominant among the hydroxy-metabolites is in line with previous studies reporting relatively high concentrations for E-10-OH nortriptyline (Edelbroek et al, 1984, Shimoda

et al, 1997, Ryu et al, 2017). The latter was not correlated to amitriptyline (or nortriptyline) concentrations, paralleling the results of Edelbroek and colleagues (1984).

In the research by Steimer et al, 2004, poor and intermediate CYP2C19 metabolisers presented lower nortriptyline to amitriptyline ratios while no significant trend towards higher amitriptyline + nortriptyline concentrations in patients with dysfunctional alleles was observed. The authors rationalise these observations whereby CYP2C19 is the main enzyme responsible for the demethylation of amitriptyline to nortriptyline, steering in opposite directions for the two compounds (Wu, 2011). Thus, CYP2C19 activity impacts on the ratio of nortriptyline to amitriptyline but not on their summed concentrations. Linden and colleagues (2008) describe a volunteer with no *CYP2C19* active alleles, having an amitriptyline demethylation ratio ([AMI]/[NOR]) over 10 times greater than the mean observed in volunteers with two active alleles.

Ryu et al, 2017, substantiate that CYP2C19 genotypes affect N-demethylation of amitriptyline into nortriptyline, with CYP2C19 poor metabolisers having highest systemic exposure to amitriptyline, regardless of variations in CYP2D6. The present research replicates the observations of these studies which were conducted on a similarly small number of subjects. In this study, even though CYP2C19 poor metabolisers were not represented in the sample (Results; section 3.3.2), genotype-inferred CYP2C19 metaboliser status characterised the significant main effect in explaining nortriptyline to amitriptyline concentration ratio variation, with intermediate metabolisers having lower mean ratios, and rapid metabolisers having higher mean ratios, compared to normal CYP2C19 metabolisers.

In the observations presented within this work (Results; section 3.3.3), the mean ratio of hydroxy-metabolites to parent was lower in patients at high risk of CYP2D6 inhibition

by concomitant drugs, compared to patients for whom the risk was conceivably inferior, and was significantly positively correlated to the *CYP2D6* activity score. This underlines the substantial role of CYP2D6 in hydroxylation, as observed by Morita et al (2000) in 41 subjects, and Halling et al (2008) in 23 subjects. In studying nortriptyline, Hodgson (2014) reports higher 10-hydroxynortriptyline to nortriptyline ratios associated with higher CYP2D6 activity. Incidentally, within the present research, the correlation between hydroxy-metabolites to parent ratios and CYP2D6 became significant when activity scores were considered. This ties well with the results of Ryu and colleagues (2017) whose observation of amitriptyline and nortriptyline hydroxylation being reduced in individuals with two decreased function alleles for *CYP2D6*, compared to those carrying one or none, was further expanded to the diplotype activity score, exposing considerable variance, particularly for *CYP2D6*10/*10*. The notion that CYP2D6 activity may vary widely within a phenotype category, particularly heterozygous vis-à-vis homozygous presentations, had already been hinted at way back by Dahl and colleagues (Dahl et al, 1996), with dosing recommendations anticipated to be more precise if based on the particular genotype, rather than a genotype-predicted phenotype (Steimer et al, 2004). Steimer and colleagues published extensively in this area fifteen years ago, with their work sustaining relevance for the explication of outcomes reported more recently in the literature, and within this research.

Steimer et al, 2004, discuss that while CYP2C19 influences concentrations of both amitriptyline and nortriptyline, variations are not expected to affect the total concentration, since altered activity shall increase one while decreasing the other. The total concentration of amitriptyline + nortriptyline is predominantly determined by CYP2D6-inflicted changes in nortriptyline concentrations. In the present study, the likelihood of the total concentration (amitriptyline + nortriptyline) being above the

expected dose-related reference ranges was observed to increase with increased risk of CYP2D6 inhibition by concomitant drugs. This risk was construed based on whether the drug was recognised as strong or weak-moderate inhibitor of the respective enzyme[69,70,71]; paroxetine being a strong CYP2D6 inhibitor was considered to denote high risk of CYP2D6 inhibition, while escitalopram, citalopram, ranitidine, and chlorpromazine denoted weak-moderate risk of CYP2D6 inhibition. The risk of CYP2C19 inhibition in the study population was related to the co-administration of omeprazole. The work of Polasek et al (2011) identified paroxetine as major perpetrator for CYP2D6-mediated pharmacokinetic drug–drug interactions; a lower level of evidence or weaker inhibition properties were documented for escitalopram, citalopram, ranitidine, and chlorpromazine in relation to CYP2D6, and for omeprazole in relation to CYP2C19.

Klieber and colleagues (2015) illustrate CYP2C19 phenoconversion after 28 days of omeprazole/esomeprazole therapy, with an 80% average lowering in CYP2C19 enzyme activity in normal and intermediate metabolisers that varied among patients with the same genotype. The impact of co-medication with CYP2D6 inhibitors has also been described as major pharmacokinetic determinant, alongside genotype, for a number of drugs in psychiatry including aripiprazole (Kiss et al, 2019), other antipsychotics (Lisbeth et al, 2016), and for nortriptyline and venlafaxine (Berm et al, 2016). Hodgson (2014) reports that patients taking nortriptyline concomitantly with CYP2D6-inhibitors had higher levels of nortriptyline and total 10-hydroxynortriptyline than those without co-medications.

[69] European Medicines Agency. Guideline on the investigation of drug interactions [Online]. CPMP/EWP/560/95/Rev. EMA; 2012 [accessed 2019 Jul 29]. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions_en.pdf.

[70] FDA. Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers [Online]. FDA; US [accessed 2019 Jul 21]. Available from: https://www.fda.gov/drugs/drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers.

[71] Mayo Clinic Laboratories. Pharmacogenomic Associations Tables [Online]. Mayo Clinic [accessed 2019 Jul 21]. Available from: https://www.mayocliniclabs.com/it-mmfiles/Pharmacogenomic_Associations_Tables.pdf.

In the exploratory exercise within the present research involving the revision of genotype-inferred CYP2D6 metaboliser status of patients at high risk of CYP2D6 inhibition by concomitant drugs, a cautious, possibly over-cautious, approach was adopted by switching the said patients to intermediate rather than poor metabolisers. However, the degree of inhibition may be dependent not only on the potency of the inhibitor, but also on the patient's allelic variants. Storelli and colleagues (2018) explained that compared to patients with two functional alleles, the rate of conversion to poor metaboliser may be higher in patients with one functional allele. Shortly after, the research group published a physiologically-based pharmacokinetic model for CYP2D6-mediated gene–drug–drug interactions prediction (Storelli et al, 2019).

The literature presents phenoconversion as a major complicating factor which is often overlooked (Shah & Shah, 2012) and under discussion in letters to the editors in response to pharmacogenetic publications (de Leon, 2015, Eikelenboom-Schieveld & Fogleman, 2018, Ziesenitz & Mikus 2019). Shah & Smith (2012, 2015) intriguingly describe phenoconversion as "a neglected entity" and "Achilles' heel" contending that the drug-induced phenomenon needs to be taken in consideration if the creditable goals of precision medicine are to be attained. In 2015, Shah & Smith elaborated further on supporting evidence that pro-inflammatory cytokines which are elevated in some inflammatory conditions may also cause phenoconversion of CYP2C19 and CYP2D6. The importance of drug-mediated phenoconversion is reiterated by Blagec and colleagues (2017) who advocate the integration of adequate algorithms to detect potential drug-drug-gene interactions within clinical decision support solutions.

The clinical scenario may present further considerations with respect to investigations, as observed in this research with respect to ECG interpretation. Since heart rate has a

biophysical effect on the QT interval, formulae, commonly Bazett's square root formula or Fridericia's cube root formula, may be used for QT correction. Bazett's prevails as the most popular route for obtaining QTc, despite Fridericia's correction possibly being more precise at the extremes of physiological heart rate (Yap & Camm, 2003), as may be the case in patients receiving tricyclic antidepressants (Robinson et al, 1982, Rochester et al, 2018). A number of published studies evaluating QTc prolongation and amitriptyline use, either specify the use of Bazett's formula in their methods, or lack details on which formula was used for QT interval correction (Castro et al, 2013, Chogle et al, 2014, da Cunha et al, 2009, Funai et al, 2014, Okayasu et al, 2012, Paksu et al, 2014, Trinkley et al, 2013, Upward et al, 1988).

Such array of data emerging in the literature and adverse events reported during the post-marketing period, as collated in the EU database of suspected adverse drug reactions (Eudravigiliance), serve to update the official sources of product information. The EU-CMDh (Co-ordination group for mutual recognition and decentralised procedures—human) published its scientific conclusions in 2015[72] with amendments to be included in the relevant sections of the amitriptyline Summary of Product Characteristics (SmPC) involving a warning on "QT interval prolongation", and "electrocardiogram QT prolonged" as a common adverse reaction. The FDA drug label for amitriptyline[73] also makes reference to cardiovascular adverse reactions and potential ECG changes.

The International Conference on Harmonisation (ICH) Guideline E14 acknowledges the controversy over the most accurate QT correction available and recommends that

[72] European Medicines Agency. CMDh Scientific conclusions and grounds for the variation, amendments to the product Information and timetable for the implementation—active substance: amitriptyline [Online]. Procedure no.: PSUSA/00000168/201501. EMA/792231/2015 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Periodic_safety_update_single_assessment/2016/10/WC500214414.pdf.

[73] U.S. National Library of Medicine. FDA Label: Amitriptyline hydrochrolide [Online]. DailyMed; NIH NLM [accessed 2019 Mar 8]. Available from: https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=1e6d2c80-fbc8-444e-bdd3-6a91fe1b95bd&audience=consumer.

corrections are performed using both the Bazett's and Fridericia's formulas, enabling detection of relevant effects on the QT/QTc interval (ICH-E14, 2005). The comparison of QT corrected with Bazett's formula (QTcB) and Fridericia's formula (QTcF) performed in this study supports research showing that the most widely adopted formula - Bazett's - underestimates at heart rates below 60 bpm and overcorrects QTc values at elevated heart rates (Fanoe et al, 2014, Luo et al, 2004, Nachimuthu et al, 2012), which may be particularly relevant for amitriptyline cases whereby Bazett's may overestimate the number of patients with QTc prolongation leading to the medication being potentially withheld as a safety measure (Vandenberk et al, 2016).

This study's observation of an abnormal ECG result being more likely in patients with high risk of CYP2D6 inhibition by concomitant drug(s), having higher mean nortriptyline concentrations, is suggestive, albeit the context allows limited extrapolation. Properties of the implicated concomitant drug(s), paroxetine in particular, may impact on the observed outcomes. Although the effects of paroxetine on the cardiovascular system are reported to be less toxic than those of amitriptyline (Hamilton et al, 1986), combinations of such drugs may increase likelihood of cardiotoxicity (Yekehtaz et al, 2013). Nortriptyline may have more pronounced effects on the elderly and children while the impact of amitriptyline appears to be more general (Goodnick et al, 2002). Ziegler et al (1977) report modest ECG changes and a weak positive correlation between pulse rate and plasma nortriptyline levels. Schneider et al (1988) correlate PR interval increases with higher nortriptyline plasma concentrations, whereas increases in QTc intervals and QRS duration were associated with increasing levels of Z-10-hydroxynortriptyline concentration. Differing cardiotoxicity for the hydroxy-metabolites was corroborated in the work by Pollock and colleagues (1992). Since CYP2D6 converts tricyclics to hydroxy-metabolites, concentrations of the latter may be elevated in ultra-rapid

metabolisers, increasing the risk for prolonged QTc intervals and QRS duration (Hicks et al, 2017). Conversely, compared to the parent compounds, the amitriptyline and nortriptyline hydroxy-metabolites, have less muscarinic acetylcholine receptor affinity, imparting negligible anticholinergic side-effects (Nordin & Bertilsson, 1995).

The observation within this research that genotype-inferred CYP2D6 and CYP2C19 metaboliser status did not correlate neither to dry mouth or drowsiness scores, nor to the total side-effect burden (Results; section 3.3.4), supports previously reported results (Hodgson, 2014, Ryu et al, 2017). Conversely, back in 2005, Steimer and colleagues did report a difference in adverse events linked to the CYP2D6 genotype, and nortriptyline, rather than amitriptyline levels, being correlated to side effects (Steimer et al, 2005). Among the effects considered in ASEC, Hodgson (2014) describes a significant association between dry mouth and hydroxynortriptyline and nortriptyline + hydroxynortriptyline concentrations. Dry mouth had previously been correlated to amitriptyline plasma levels by Gupta and colleagues (1999) who found no relationship between nortriptyline plasma concentrations and anti-cholinergic effects. Of these effects, dry mouth, but not drowsiness, correlated to amitriptyline levels (Gupta et al, 1999). The latter observations are comparable to the results here presented. In view of the heterogeneity in the few populations studied, doses, time course, and results, associations are difficult to construe in a determinant way.

Albeit probably outside the remit of pharmacogenetic implications, it is worth referring to the CHMP 2017 amitriptyline assessment report[74] which notes that slow titration of amitriptyline doses shall alleviate severity of sedation and dry mouth, among other side-

[74] European Medicines Agency. Assessment report Referral under Article 30 of Directive 2001/83/EC [Online]. Procedure number: EMEA/H/A-30/1430. CHMP; EMA/255467/2017 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Saroten_30/WC500227970.pdf.

effects, and increase tolerability. This explains the rationale of recommending a markedly low starting doses. The observations within this research indicate that sedation may indeed become less problematic in the long-term, even if there is dose escalation. In this study, it has further been observed that in the case of dry mouth, it persists over months of amitriptyline use and is perceived to interfere sufficiently in the patients' quality of life to merit reporting. This may question the notion that anticholinergic adverse effects of amitriptyline generally abate with continued treatment, and favours the consideration that anticholinergic symptoms may fluctuate in their occurrence (Bryant et al, 1987) or tolerance does not necessarily develop during long-term medication (Giller et al, 1985). It was outside the plan of this research to perform methodical causality assessment, either for prolonged-QT, or any of the adverse reactions reported by the patients, since the study design did not involve pre-treatment baseline. Thus, potential pre-existing causality or coexistent disease/drug causality cannot be systematically excluded.

As discussed by Hodgson (2014), evidence is more mature with regards to the relationship between CYP genotypes and serum concentrations, rather than clinical outcomes. In turn, the evidence supporting increased risk of side-effects among CYP2D6 poor metabolisers, as presented in CPIC guidance, may have less robust backing. It can be concluded that this work, particularly the integrated approach adopted in the construal of amitriptyline genotype-guided dosing recommendations for recruited subjects under psychiatric care, provides supporting evidence to the understanding that due consideration of contributors to a patient's metabolic profile at a point in time, may complement the acclaimed role of pharmacogenetics in delivering precision medicine.

4.2 Methodological critique and study limitations

The methodology necessitated keeping up with ongoing progress around the three distinct aspects amalgamated into a coalesced outcome. During the course of this research, the literature in the sphere of pharmacogenetics concerning psychiatry matured at a remarkable pace amid efforts towards standardizing terms for clinical pharmacogenetic test results (Caudle et al, 2017), amendments in genotype-phenotype interpretations (CPIC, 2019), and a breadth of publications expanding on the state of play (García-González et al, 2017, Abbasi, 2018, Stern et al, 2018). The concepts involved in such developments were in line with the research questions posed.

The 2017 revisions to the amitriptyline Summary of Product Characteristics (Results; section 3.1.1), expounding on the potential influence of CYP2D6 and CYP2C19, are an example of developments taking place during this study. Besides valuing the harmonisation endeavours in the regulatory ambit, resources from the competent authority were utilised in this research to assess pharmacogenetic considerations in the evaluation of safety concerns (Results; section 3.1.2). The static Proportional Reporting Ratio (PRR) extracted from the EudraVigilance Data Analysis System of the European Medicines Agency for the evaluation of amitriptyline as main drug of interest, and suspected drug interactions reports, is a measure of association and not causality, implying that some of the signals identified may correspond to events that are unrelated to treatment (Evans et al, 2001). Although statistical association may reflect a causal relationship between exposure to a medicine and the occurrence of an adverse event, numerous factors, including concomitant medications may have an important influence. Reproducible measures of the relationship-likelihood in ADR cases are hindered by divergences in the thresholds chosen for the PRR and for the count of drug-event

combination reports (Slattery et al, 2013) and by the unavoidable subjectivity of judgements which challenge the standardization of causality assessment systems (Hire et al, 2013). Causality assessment in this research was restrained by the equivocal nature of the case reports – a limitation which was analogously encountered when considering Individual Case Study Reports (ICSRs) for dry mouth and sedation. Reactions representing the same phenomenon may be reported under different terms which may obscure the true effect and could present as a limitation to this study. It is apt to have effects such as sedation, somnolence and drowsiness ordinarily grouped into a single adverse reaction to avoid dilution,

In view of the numerous variables under study, interpretation of the data and compilation into logically presented outcomes is complex. The inclusion of patients from the pain management setting facilitated evaluations along the amitriptyline dosing continuum, including doses at the lower end, which are typically prescribed at initiation of therapy. A more homogenous group, particularly with respect to therapeutic indication, amitriptyline dosing strategy and chronology, may allow more straightforward analyses, although the comparative advantage of a naturalistic setting as studied in this research would be diminished.

Standard methods of analysis such as HPLC presented their limitations when the exigencies of the research required separation of closely related metabolites and low levels in serum. This led to the development of the practical LC-MS/MS method for rapid simultaneous determination of amitriptyline, nortriptyline and the hydroxy-metabolites in human serum. The LC-MS/MS methodology, using a state-of-the-art instrument, was utilised for the analysis of sera from recruited patients receiving between 10 and 175mg amitriptyline daily. Despite the newly developed method having an improved lower limit

of quantification compared to the discussed analytical methodologies with a similar purpose, there were still sporadic cases in which measured concentrations were below the validated LLOQ, < 0.5 ng/mL, particularly for the hydroxy-metabolites. Notwithstanding, it is envisaged that any erroneousness in levels so low is most likely inconsequential. While encompassing the hydroxy-metabolites, which are often not catered for in standard therapeutic drug monitoring, the analysis did not consider all potential minor metabolites, such as desmethylnortriptyline. Serum concentration levels bordering closely on the margins of the dose-related reference ranges may well fit within the expected range on account of marginal inter-laboratory discrepancies in the reported measurements. It is construed that the analytical techinique developed in this research is useful for clinical practice purposes since concentrations of particular clinical relevance deviate substantially from the predicted range, as portrayed by the analyses linking difference in measured concentration from expected amitriptyline + nortriptyline range to the risk of CYP inhibition by concomitant drugs, and genotype (Results; section 3.3.3).

Fan & Bousman (2019) discuss the important implications that genotype-guided dosing recommendations have in the field of psychiatry whereby 38% of gene-drug pairs with dosing guidelines are relevant to psychiatric therapy. It is highlighted that the said guidelines may only facilitate clinical implementation of pharmacogenetics if test results can be made available. Their analysis of 22 commercial pharmacogenetic test panels confirmed that the *CYP2D6*-amitriptyline and *CYP2C19*-amitriptyline gene-drug pairs were included in all tests (Fan & Bousman, 2019). In the research here presented, genotyping congruently considered *CYP2D6* and *CYP2C19*, whereas the possible impact of the involvement of other CYPs such as *1A2*, *2C9*, *3A4*, was not investigated since presumed to be play minor roles at therapeutic doses (Olesen & Linnet, 1997, Venkatakrishnan et al, 1998). Although a discrete set of alleles were tested, some of the

tested patients could have undetected alleles and structural variants, which may affect the observed frequencies, particularly in the case of *CYP2D6,* having over 100 allelic variants and subvariants, that amplify the complexity for genotyping and interpretation (Cavallari et al, 2019).

Gaedigk and colleagues (2017) report that the *CYP2D6*10* decreased-function allele is highest in East Asians (45%) and lowest in Oceanians and Europeans, averaging 1.6 and 2.6 %, respectively. Indicatively, certain **10*'s identified in the population tested within this research may be misclassifications of *CYP2D6*36* resulting as a single entity with *CYP2D7*-derived exon 9 conversion or as a hybrid switch to *CYP2D7*. The documented **36* structural variation denotes no function, compared to decreased function for **10*. Accordingly, Del Tredici and colleagues (2018) discuss that frequencies for *CYP2D6 *10* vary substantially, depending on the test capability of detecting specific structural variation. Compared to normal metabolisers, intermediate and poor metabolisers tend to present more structural variants, and are therefore at increased risk of misclassification. This may warrant a broader test panel of alleles that includes structural variants, to enable more accurate CYP2D6 genotyping and prediction of phenotype (Del Tredici et al, 2018).

Although the size of the case-based sample cohort studied in this research may not be optimal for generalizing inferences from auxiliary observations related to allele/diplotype/phenotype frequencies, it is appropriate to note that patients with a CYP2C19/CYP2D6 metaboliser status that deviates from the normal, may be under-represented in the sample population, since empirical identification by their clinicians or adverse outcomes experienced, may lead to amitriptyline withdrawal and deterred chances of being recruited. The risk of patients on tricyclic antidepressants for early discontinuation and switching to another antidepressant may be influenced by CYP

metaboliser status (Bijl et al, 2008, Peñas-Lledó et al, 2013). Sample size is a substantial limitation in this research work, while other limitiations are recognised. Notwithstanding that participants were asked about compliance and to list all medications being taken, there is always the possibility of non-disclosure, which may amplify phenotype-genotype discordance or add to the variability between similarly characterized individuals. The recruitment protocol included patients over 18 years of age and the observations may not be generalizable to paediatric populations including children and adolescents. The antidepressant pharmacogenetics knowledge base is still emerging in paediatrics and further research is warranted, although upshots thus far echo much of the gene-antidepressant associations characterised in adult populations (Maruf et al, 2019).

While none of the recruited subjects reported hepatic or renal comorbidities, routine bloods identified sporadic cases of wavering bilirubin, alkaline phosphatase, GGT, ALT, urea and creatinine levels, which may be indicative of sub-clinical conditions, such as cholestasis. The possibility of such issues influencing the outcomes cannot be totally ruled out (Lieberman et al, 1985, Lauschke & Ingelman-Sundberg, 2016). Furthermore, the protein binding of tricyclic antidepressants may be increased in cardiac patients (MacKichan & McGory, 2009). No substantial cardiac comorbidities were documented for the recruited subjects, some of whom reported receiving treatment for hypertension. It is worth noting that, in view of the heterogeneous setting and lack of baseline measurements, ECG examination outcomes were not intended and cannot be extrapolated to determine whether amitriptyline (or aberrant levels of its metabolites) cause QT prolongation, which, although representing a recent safety warning added to the SmPC, is rather controversial. Investigating QTc prolonging effects of TCAs in a sub-group from the Rotterdam study, Noordam and colleagues (2015), demonstrated statistically significant QTc prolongation with amitriptyline, using Bazett corrected QTc interval,

which was lost upon adjusting for the increase in heart rate. The authors inferred that Fridericia's formula might be preferred and suggested prospective revision to the warnings put forward by regulatory bodies in that TCAs might not indeed be associated with QTc prolongation. The 2018 review by Rochester et al (2018) included mixed studies conducted in neuropathic pain in which no significant impact on QTc was seen (da Cunha, 2009) or amitriptyline was reported to have significantly prolonged the QTc interval (Funai et al, 2014), although to a lesser extent than observed with doses used in depression. Caution is recommended in generalizing data, with practical distinction between studies on QT prolongation in overdose or toxicity, as opposed to standard clinical use. This research substantiates that the method of QT correction may be critical in the interpretation of the data. Gender, age and confounding medical conditions or medications are also known to impact on QT prolongation (Rochester et al, 2018).

Recruited subjects were also assessed using MADRS and PainDetect at one point in time, and no conclusions on the efficacy of amitriptyline doses may be inferred, with the study of treatment response being outside the scope of this research. As for the side-effects reported, patients were asked to indicate whether they attribute the reported effect to amitriptyline therapy, or otherwise. The underlying disease state and co-administered drugs may still impart confounding effects. Side-effect measures were considered as a quantitative trait prior to dichotomising in reported/not reported to mitigate potential loss of statistical power for variables likely to contribute small effect sizes.

A recent multi-site investigation by Cavallari and colleagues (2019), embodies the methodological considerations also confronted in this study. Major challenges identified and reflections include: choice of non-invasive method for DNA sample collection since phlebotomy may not be available in all settings; the ambiguity in phenotype assignment

when genotyping is unable to identify CNV and determine which allele is duplicated in *CYP2D6* heterozygous genotypes, with most sites resorting to a ranged phenotype; and phenoconversion potential with concomitant CYP inhibitor use. The authors discuss technical issues, mainly related to the complexity of the *CYP2D6* gene, which may explain why CYP2D6 genotyping is typically implemented in practice subsequent to CYP2C19. It is positive to note that while assistance in prescribing antidepressants was a common rationale for ordering CYP2D6 testing, genotype results were applied to multiple therapies by most institutions (Cavallari et al, 2019).

4.3 Practical implications and future directions

The World Health Organisation[75,76] reports that despite numerous treatment options, unipolar depressive disorder ranks as the leading chronic condition in Europe, accounting for 11 percent of all years lived with disability. Rates of employment may vary from 18 to 30 percent and compared to the general population, the life expectancy is 20–30 years lower. Advancements resulting in auspicious trends in the management of diseases within the general population, such as diabetes and ischaemic heart disease, have not equally addressed quality of life and mortality in those suffering from mental illness. The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) trial demonstrated that after initial treatment, only a third of subjects attained remission, with the rate declining further with an increasing number of treatment trials (Rush et al, 2006).

[75] World Health Organization. Mental Health [Online]. WHO [accessed 2018 Jul 31]. Available from: http://www.euro.who.int/en/health-topics/noncommunicable-diseases/mental-health.

[76] World Health Organisation. The European Mental Health Action Plan 2013–2020 [Online]. WHO [accessed 2018 Jul 31]. Available from: http://www.euro.who.int/__data/assets/pdf_file/0020/280604/WHO-Europe-Mental-Health-Acion-Plan-2013-2020.pdf.

Healthcare systems and medical practitioners act abidingly to ensure that patients receive treatment for cancer or heart disease. Depression, on the other hand, is marked by doubts and controversy, with the authors of the Lancet 2018 review (Cipriani et al, 2018) warning that only one in six patients suffering from depression are receiving treatment. The ones that are prescribed medication may not necessarily pull through the risk-benefit aggregate of antidepressants. Lozupone et al (2016) underline the prevalence of therapeutic failures and clinical worsening of symptoms attributed to adverse reactions among older patients receiving psychiatric treatment. The review highlights the relevance of pharmacogenetic data, mainly related to the cytochrome P450 enzyme family, in understanding how genetics, together with environmental and physiological factors model the phenotype observed with advancing age. Awareness of altered metaboliser status is proposed to assist clinical decisions by limiting therapeutic attempts, avoiding interacting therapies and promoting drug safety.

Pharmacogenetics impacts pharmaceutical companies through its role in drug discovery, diminishing drug attrition in trials, categorising drug responders, and drug safety during medicinal product development and post-marketing (Pushpakom & Pirmohamed, 2012). The search for genetic biomarkers that may influence drug safety tends to be more clear-cut during the drug development phase. A case-control study design may be conducted, genotyping both patients with ADRs (cases) and patients without ADRs (controls), and the frequencies compared. Conducting such studies on an established drug, as is the case with amitriptyline, may be perceived less exigent. ADRs reported to the regulators may equally generate alarm signals for potential drug safety risks. Multiple drugs for which pharmacogenomic guidelines are available may be co-administered in up to a third of patients, with higher incidence in patients over forties where approximately half of patients receive at least one such drug (Samwald et al, 2016). The CYP2D6 enzyme

metabolises approximately forty percent of drugs recurrently cited in adverse drug reaction studies (Phillips et al, 2001). Nonetheless, insufficient reporting and non-granulated information in the submissions are recognized flaws in the undemanding disposition of the ADR reporting system. Reflections that arise from the implications identified in this study point towards a missing link with respect to pharmacogenetic considerations across safety surveillance.

La Russa and colleagues (2017) describe the experience within an Italian teaching hospital which highlights the potential of pharmacogenomics in diminishing adverse reactions and encourage further pharmacogenomic trials for the personalisation of pharmacotherapy, as well as the refinement of current pharmacovigilance structures. Systematic evaluation of the ADR risk conferred by specific genetics requires transnational co-operation and incentives, as well as concordance on the part of healthcare professionals and patients (OECD, 2009). Active ADR surveillance through dedicated clinicians, trained to identify, investigate and report ADRs, represents a targeted approach which has proved successful in the Canadian paediatric practice. The Canadian Pharmacogenomics Network for Drug Safety (CPNDS) identified genetic factors causing codeine-induced neonatal opioid toxicity and cisplatin-induced hearing loss (Shaw et al, 2011). Research on the more widespread adoption of such surveillance systems, encompassing a multi-disciplinary group from regulatory, analytical and clinical fields, may allow pharmacogenetics to elucidate on the safety implications for drugs in use in our day.

Once consolidated data is at hand, making it uniformly accessible to the ones at the foreground of the scenario who may foster, or otherwise, its application in the day-to-day clinical practice, can be a major contribution to translational pharmacogenetics. The

response of regulatory sciences to the dynamic progression of pharmacogenetics is remarkable and outcomes are enrolling in real time with respect to the integration of genomics throughout the life-cycle of new drugs. While the support of the regulatory infrastructure towards integrating pharmacogenetics in official product information may seem ineffectual at the outset, thorough consideration of the multiple factors implicated in the translational quality of the field, may not corroborate early hopes of having a specific genetic test to provide definite prediction of the clinical picture. This may explain potential reluctance in categorically quantifying genotype-based dose alterations within product labelling. The inclusion of reference to CYP2D6 (more explicitly than CYP2C19) may be tantamount to the point that therapeutic drug monitoring to guide amitriptyline therapy is mostly based on the total concentration of amitriptyline and nortriptyline, which, as discussed in this work, is primarily influenced by CYP2D6.

By directly measuring blood drug concentrations, testing both *CYP2D6* and *CYP2C19*, and also consider concomitant drugs, this research attempted to limit confounding factors while exploring potential additive effects. The inclusion of patients, analogously on amitriptyline therapy, from two recruiting arms – psychiatry and pain – portended comparative inferences with respect to the difference in the range of doses administered. The EU-SmPC prescribing information for amitriptyline in the EU, unlike the FDA label, includes 'treatment of neuropathic pain in adults' as a therapeutic indication. The 2017 CHMP assessment report[77] notes that twice daily dosing may be necessary for immediate release formulations, to limit sedation and ensure a 24-hour therapeutic coverage, possibly recognizing that the blood concentrations resulting from low-dose amitriptyline administered once daily potentially verge on sub-therapeutic. The latter may be

[77] European Medicines Agency. Assessment report - Referral under Article 30 of Directive 2001/83/EC [Online]. Procedure number: EMEA/H/A-30/1430. CHMP; EMA/255467/2017 [accessed 2019 Jul 28]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Saroten_30/WC500227970.pdf.

particularly relevant for the 10 mg starting dose, which prescribers might feel most confident to adhere to over the proposed 2–4 weeks in which efficacy may be assessed, even though in the studies implicated to support the use of amitriptyline in the treatment of neuropathic pain, a 10 mg daily dose is underobserved (Boyle et al, 2012, Graff-Radford et al, 2000, Leijon & Boivie, 1989, Max et al, 1988, Max et al, 1992, Mishra et al, 2012, Rintala et al, 2007, Rowbotham et al, 2005, Vrethem et al, 1997).

As the pharmacogenetics knowledgebase, along with data on CYP inhibition profiles and interactions, continues to expand, it is compelling to progressively reconsider the tricyclic antidepressants (Gillman, 2007). Researchers are especially enthused in recognising that only one in three pain patients may be considered to be a responder to amitriptyline's co-analgesic effect[78]. A Phase IV clinical trial - *Effects of Amitriptyline on Central Pain Processing in Healthy Volunteers Depending on CYP Pharmacogenetics*[79] - was launched in 2014, with a target sample size of forty-eight participants, to study the implications of *CYP2D6* or *CYP2C19* variants. Upon contact with the investigators from Bern University Hospital leading this research, it was conferred how results, initially expected for the beginning of 2018 were as yet unavailable. The investigators discussed how difficulties in recruiting CYP2D6 ultra-rapid metabolisers led them to consider performing a preliminary analysis to assess the way forward.

Recruitment is an issue in most research settings, but more so when the setting involves genetics and psychiatry. Healthcare professionals have the academic and experiential background to be entrusted with patient treatment but for research in these areas, policy-makers are somewhat wary. The Malta Mental Health Act (2012) requires that capacity

[78] National Library of Medicine. Genetic Determinants of Amitriptyline Efficiency for Pain Treatment [Online]. National Institutes of Health [accessed 2018 Sept 2]. Available from: https://clinicaltrials.gov/ct2/show/NCT02256943.
[79] World Health Organisation. International Clinical Trials Registry Platform Search Portal [Online]. WHO [accessed 2018 Sept 2]. Available from: http://apps.who.int/trialsearch/Trial2.aspx?TrialID=NCT02256943.

is certified by an independent specialist for research involving persons with mental disorders. One may perhaps question whether this is safeguarding vulnerable patients or creating orphan populations by amplifying distress in areas where new research is to be encouraged. Unease was evident in patients within this study at the awareness that their capacity required third-party confirmation. Subjecting prospective participants to ancillary assessments that can trigger fear of institutionalisation or repercussions, may hinder reliable data collection and delay translational research. Incorporation, for example in pharmacy curricula, of recognized training on the evaluation of decision-making impairment, pursuant to the type and phase of mental disorder may enable capacity and informed consent in pharmacist-led psychiatric clinical studies to be construed as an ongoing pharmacist-patient appraisal. This could lessen patient distress which may arise from inflicting overzealous practices and be a consensus alternative to the requirement of an independent specialist.

Other than the recruitment hurdles, the exploration of pharmacogenetics is known to be intricately marked by the complexity of mechanisms underlying drug response, lack of data standardisation and quantitative understanding of the information available, limited knowledge vested in patients and healthcare professionals, scattered policies and insufficient funding (Sadée & Dai, 2005, Horgan et al, 2014). Practical considerations, such as time, provider burden and pharmacoeconomics, although not within the scope of this work, may certainly influence the implementation prospects of the approach discussed. For example, just the cost of sampling, genotyping and monitoring blood levels for the recruited subjects approximated to 170 Euro per patient - a factor that needs to be put in the cost-benefit analysis. With respect to the clinical associations, the subjective experience of the disorders for which amitriptyline is administered, is likely to complicate interpretation of results.

For tricyclic antidepressant drugs, literature review points towards the effectiveness of therapeutic drug monitoring in view that pharmacokinetically dosed patients tend to be discharged earlier from hospital than the empirically dosed patients (Hiemke et al, 2018). Pharmacogenomic-guided therapy is also reported to outperform the treatment-as-usual approach. Pre-therapeutic genotyping is generally expected to be of most benefit to individuals with extreme pharmacokinetics, whose prevalence is low and cost-effectiveness is difficult to demonstrate (Steimer et al, 2004). The revival of a well-established drug, with advantageous efficacy and cost, may provide better economic justification for genotyping than merely identifying poor and ultra-rapid metabolisers. Steimer et al, 2005, claim that the majority of patients tolerate standard-dosage amitriptyline therapy very well with few side effects. Two thirds of patients may receive standard doses of amitriptyline, whereas 'high-risk' patients could receive modified doses of amitriptyline or treatment with newer, more expensive drugs.

The cytochrome P450 superfamily of hemoproteins catalyzes the majority of phase I reactions. Even though representation of total CYP content in the liver may be low for some variants, for instance around 2-5 percent in the case CYP2D6, the role played in drug metabolism can be substantial (Pinto & Dolan, 2011). CYP450 enzymes, particularly CYP2D6 and CYP2C19, regulate the metabolism of approximately 25 percent of all prescription drugs (Jain, 2005), implying that the result of one pharmacogenetic test is likely to also be useful for future treatments. Pharmacogenetic testing is associated with prospective savings of direct and indirect costs and improved quality of life, in managing major depressive disorder patients non-responsive to previous treatment (Hornberger et al, 2015), moderate-to-severe depression and/or anxiety (Najafzadeh et al, 2017) and in chronic pain (Morlock & Braunstein, 2017), with

genotyping-based treatment expenditures being potentially offset by reduced utilization of medication and costs related to adverse events.

Availability and consistency of test results and their socio economic impact are factors intrinsic to pharmacogenomic data which together with extrinsic factors, such as awareness of the medical profession, characterise potential reasons for the poor uptake of genetic testing in practice (Prasad, 2009). Point-of-care methodologies may prove more pragmatic in clinical application, providing rapid results at the patient's bedside, albeit testing may be more expensive compared to laboratory-based genotyping assays (Wirth et al, 2016). The latter work by Wirth, undertaken during a PhD within the Department of Pharmacy, University of Malta, highlighted the practicality of point-of-care CYP2C19 genotyping in patients prescribed clopidogrel therapy.

Barriers on the economic level are entangled in the process since reimbursement is insufficient or non-existent (Horgan et al, 2014), in the absence of a unified approach on how to value precision medicine. Health technology assessment (HTA), through networks among member states, such as EUnetHTA, could collaborate to facilitate information exchange, advise on evidence related to risk-benefit balance and value, as well as economic impact of the use of personalized medicines (Nofziger et al, 2014). Since 2010, the European Medicines Agency has embraced a project for scientific advice in parallel with HTA bodies that enables medicines developers to obtain feedback from HTA bodies and regulators simultaneously, for instance, with respect to companion diagnostics (Ehmann et al, 2014). 'Biomarkers on a roll', a 2010 Editorial in Nature Biotechnology[80], pointed towards trial inadequacies, arbitrary biomarker research, lack of validation procedures, failure to characterize efficacy and toxicity in pharmacogenetics

[80] Editorial. Biomarkers on a Roll. *Nat Biotechnol* 2010;28:431. doi:10.1038/nbt0510-431.

terms and absence of an established scientific framework as major contributors to the slow progress of genotype-based personalised medicine. Meanwhile, potential legal repercussions also started emerging for marketing a drug without warning of genetic variants that may predispose to adverse outcomes[81].

Research barriers often portend barriers in evaluating clinical utility and tangible implementation which have been widely exposed in the literature (Agúndez et al, 2012, Malhotra et al, 2012, Tremblay & Hamet, 2013, Altar et al, 2015, Arwood et al, 2016, Dawes et al, 2016, Preskorn, 2016, Lazaridis, 2017, Lindor et al, 2017, van der Wouden et al , 2017, Weinshilboum & Wang, 2017, Ahmed et al, 2018, Dressler et al, 2019). The literature is also expanding on the experience of healthcare professionals with pharmacogenetic testing (Moaddeb et al, 2015, Vassy et al, 2018) particularly in primary care and community settings. The healthcare professional educational aspect of pharmacogenomics is gaining momentum, extending from Mayo Clinic in Minnesota (Formea et al, 2013) to the University of Patros in Greece (Patrinos & Katsila, 2016), with pharmacogenetics competency in pharmacy practice anticipated to develop clinical utilization strategies (Roederer et al, 2017).

As an offshoot of research, such as the present project, students are exposed to practice-based examples that empower confidence in pharmacists applying clinical pharmacogenetic models. Stimulation of hands-on thinking to apply basic principles in practice, serves to expose the strong points, as well as the necessitated developments, for prospective clinical implementation of pharmacogenetics, spearheaded by pharmacists. A major weakness identified relates to limitations within the IT framework, particularly

[81] Department of the Attorney General, Hawaii. News Release 2014-09: Attorney General files suit against manufacturers and distributors of the prescription drug Plavix [Online]. Hawaii [accessed 2019 Jul 28]. Available from: http://ag.hawaii.gov/wp-content/uploads/2014/01/News-Release-2014-09.pdf.

with respect to electronic health records, clinical decision support structures and communication strategies, which necessitate further research. While making results and interpretation notes available directly to the clinicians on a case-by-case basis, highlighting the data of relevance to avoid alert fatigue, may form a sensible basis for future investigations, it is common practice for patients to have their treatment plan revised by practitioners outside the hospital setting and the relevant data may become fragmented and not completely accessible.

Demonstrating the benefits of pharmacogenetics in real-world settings gives us an opportunity to support incorporation into routine clinical care. Personal genome testing may be offered to students, and healthcare professionals, to induce enthusiasm and develop first-hand understanding. The development of interactive educational models, practice-based examples and possibly 'train-the-trainer' initiatives shall support healthcare professionals to accurately apply pharmacogenetic data to drug-therapy selection, dosing, and monitoring. This engagement in pharmacogenetics may expand further by establishing specific interdisciplinary networking fora to mitigate potential threats.

The developments endorsed during the course of this research, such as the latest consensus on CYP2D6 genotype-phenotype interpretation, augur enhanced standardisation within pharmacogenetic studies. Clinical research with outcomes that may be unequivocally compared is anticipated, albeit specific factors, such as ethnicity, still necessitate careful consideration. A number of other drugs apart from the ones listed in the amitriptyline SmPC are known for their implications on the isoenzymes involved in the metabolism of amitriptyline (Badyal & Dadhich, 2001), and which may potentially be the culprit in drug interaction reports.

Combining drug interaction information with insights from genotyping, actually measured metabolic phenotypes and therapeutic drug monitoring, as may be applicable, should enable identification of individuals for whom consequences of interactions and genetics pose superior risk than would be predictable were either considered in isolation (Shah et al, 2016, Sugarman et al, 2016). Although risk analysis may seem trivial when measured concentrations are still distant from the toxic range, potential implications of phenoconversion may become particularly clinically relevant on dose titration, which is common practice in amitriptyline therapy. While polymorphisms may have most significant impact in cases of poor metabolism, the influence of a CYP perpetrator drug may be particularly noteworthy in normal metabolisers. Interactions are likely to be dependent on genotype, as well as the properties of the drugs themselves, such as whether metabolites have comparable effects to parents, and whether minor metabolic pathways or transporters for the respective drugs are also disrupted (Bahar et al, 2017). Recognising that, compared to drug-drug interactions alone, drug-gene interactions and drug-drug-gene interactions are reported to augment the quantity of interactions by approximately 50% (Verbeurgt et al, 2014), caution with generalisations is recommended for future studies evaluating this domain.

Gaedigk and colleagues (2017) discuss the anticipated benefits from undertaking 'genotype-stratified pharmacokinetic studies for *high-priority drugs*'. The latter opens another Pandora's box. Back in 1997, Andrew Marshall, featuring in *Nature Biotechnology*, discussed that while pharmacogenomics may interest large pharmaceutical companies to revive, or seek new indications for existing drugs, big pharma is more likely to invest in new drug development programmes, with the pharmacogenomic refinement of older drugs being possibly adopted by smaller companies or research groups (Marshall, 1997). While Skykiotis and colleagues trace the

roots of today's medicines, personalized on the basis of genome, to Hippocratic teachings (Sykiotis et al, 2005), Schaffner in 2010 dived deep into philosophical perspectives of the person seeking psychiatric therapy, in the light of personalised medicine – the body which may host variations in metabolism, and the mind within a complex familial and social context (Schaffner, 2010). Lakhan and colleagues (2010) caution that biomarker testing may be prohibitively expensive and resource-intensive, and its application to multi-factorial psychiatric disorders, may have discriminatory repercussions, such as in health insurance and employment (Lakhan et al, 2010). Discussions have also ensued on whether pharmacogenetics is intended to meet the needs of prosperous nations, or is to translate in a 'luxury', disputed as necessary or unnecessary, for global public health (Olivier & Williams-Jones, 2011). Ethical concerns, implicating personal responsibility and disruptive justice, are triggered by the predictive aptitudes of this area of study (Gefenas et al, 2011). It is hoped that amid all contentions, psychiatric treatment is appraised and not shunned.

4.4 Conclusion

Robust evidence on the use of amitriptyline, and other established drugs, which typically emerges from randomized controlled trials, is scant for patients having aberrant metabolic profiles. Clinical outcomes are determined by how the drug is used in everyday practice, which presents an opportunity for real-world evaluation of patients with complex comorbidities and co-medications. Integrating pharmacogenetics within a proactive practice for the reporting of adverse drug reactions, should provide effective means to further understand the contribution of genetic factors as risk-minimisation measures.

This research illustrates the multiple considerations that are entailed in establishing patient-tailored pharmacotherapy. Developments on analytical aspects are presented, while it is construed that even implementation of a comprehensive testing strategy will have its weaknesses unless other confounding factors, such as the concurrent administration of CYP inhibitors, are considered. The observations portray how, independently of a therapeutic reference range, therapeutic drug monitoring and dose-related reference ranges, may be used to identify potential pharmacokinetic abnormalities which impact on a patient's systemic exposure to amitriptyline, as the example under study. Blood levels outside expected ranges may serve as an alert to actively look for gene polymorphisms or drug interactions. This project provides supportive evidence on the practical application of evolving research outcomes and recommendations to better understand clinical presentations.

Further work is necessitated in developing the areas elucidated by this research. Progress in generating individualized data, shall mitigate the hesitation associated with trial-and-error prescribing and drive informed clinical judgement, coupled with sensible investigations, embracing the expedient implications of pharmacogenetics.

References

Abbasi J. Companies tout psychiatric pharmacogenomic testing, but is it ready for a store near you? *JAMA* 2018;320(16):1627-9.

Agúndez JAG, Abad-Santos F, Aldea A, Alonso-Navarro H, Bernal ML, Borobia AM, et al. Toward a clinical practice guide in pharmacogenomics testing for functional polymorphisms of drug-metabolizing enzymes. Gene/drug pairs and barriers perceived in Spain. *Front Genet* 2012;3:273. doi:10.3389/fgene.2012.00273.

Ahmed AT, Weinshilboum R, Frye MA. Benefits of and barriers to pharmacogenomics-guided treatment for major depressive disorder. *Clin Pharmacol Ther* 2018;103(5):767-9.

Altar CA, Carhart J, Allen JD, Hall-Flavin D, Winner J, Dechairo B. Clinical utility of combinatorial pharmacogenomics-guided antidepressant therapy: Evidence from three clinical studies. *Mol Neuropsychiatry* 2015;1:145-55.

Aro AL, Anttonen O, Kerola T, Junttila MJ, Tikkanen JT, Rissanen HA, et al. Prognostic significance of prolonged PR interval in the general population. *Eur Heart J* 2014;35(2):123-9.

Arwood MJ, Chumnumwat S, Cavallari LH, Nutescu EA, Duarte JD. Implementing pharmacogenomics at your institution: Establishment and overcoming implementation challenges. *Clin Transl Sci* 2016;9(5):233-45.

Ashour S, Kattan N. Simultaneous determination of nortriptyline hydrochloride and fluphenazine hydrochloride in microgram quantities from low dosage forms by liquid chromatography–UV detection. *J Pharm Anal* 2012;2(6):437-42.

Avramopoulos D. Genetics of psychiatric disorders: Methods: Molecular approaches. *Psychiatr Clin North Am* 2010;33(1):1–13.

Badyal DK, Dadhich AP. Cytochrome P450 and drug interactions. *Indian J Pharmacol* 2001;33:248-59.

Bahar MA, Setiawan D, Hak E, Wilffert B. Pharmacogenetics of drug-drug interaction and drug-drug-gene interaction: a systematic review on CYP2C9, CYP2C19 and CYP2D6. *Pharmacogenomics* 2017;18(7):701-39.

Bandelow B, Baldwin D, Abelli M, Altamura C, Dell'Osso B, Domschke K, et al. Biological markers for anxiety disorders, OCD and PTSD – a consensus statement. Part I: Neuroimaging and genetics. *World J Biol Psychiatry* 2016;17(5):321-65.

Bank PCD, Caudle KE, Swen JJ, Gammal RS, Whirl-Carrillo M, Klein TE, et al. Comparison of the guidelines of the Clinical Pharmacogenetics Implementation Consortium and the Dutch Pharmacogenetics Working Group. *Clin Pharmacol Ther* 2018;103(4):599-618.

Barbui C, Hotopf M. Amitriptyline v. the rest: still the leading antidepressant after 40 years of randomised controlled trials. *Br J Psychiatry* 2001;178:129–44.

Bate A, Evans SJ. Quantitative signal detection using spontaneous ADR reporting. *Pharmacoepidemiol Drug Saf* 2009;18(6):427-36.

Baumann P, Jonzier-Perey M, Koeb L, Küpfer A, Tinguely D, Schöpf J. Amitriptyline pharmacokinetics and clinical response: II. Metabolic polymorphism assessed by hydroxylation of debrisoquine and mephenytoin. *Int Clin Psychopharmacol* 1986;1(2):102-12.

Beck AT, Ward CH, Mendelson M, Mock J, Erbaugh J. An inventory for measuring depression. *Arch Gen Psychiatry* 1961;4:561-71.

Bergés R, Sanz-Nebot V, Barbosa J. Modelling retention in liquid chromatography as a function of solvent composition and pH of the mobile phase. *J Chromatogr A* 2000;869:27-39.

Berm E, Kok R, Hak E, Wilffert B. Relation between CYP2D6 genotype, phenotype and therapeutic drug concentrations among nortriptyline and venlafaxine users in old age psychiatry. *Pharmacopsychiatry* 2016;49(5):186-90.

Bertilsson L, Dahl ML, Dalén P, Al-Shurbaji A. Molecular genetics of CYP2D6: Clinical relevance with focus on psychotropic drugs. *B J Clin Pharmacol* 2002;53(2):111–22.

Bijl MJ, Visser LE, Hofman A, Vulto AG, van Gelder T, Stricker BH, et al. Influence of the CYP2D6*4 polymorphism on dose, switching and discontinuation of antidepressants. *Br J Clin Pharmacol* 2008;65(4):558-64.

Black JL. Tamoxifen and irinotecan pharmacogenomics. In: Highsmith WE, editor. Molecular Diagostics: 12 tests that changed everything, molecular and translational medicine. New York: Springer;2014. p. 35-54.

Blagec K, Kuch W, Samwald M. The importance of gene-drug-drug-interactions in pharmacogenomics decision support: an analysis based on Austrian claims data. *Stud Health Technol Inform* 2017;236:121-7.

Blum M, Grant DM, McBride W, Heim M, Meyer UA. Human arylamine N-acetyltransferase genes: Isolation, chromosomal localization, and functional expression. *DNA Cell Biol* 1990;9:193–203.

Bonke G, Jensen BP. Fast and sensitive LC–MS/MS assay for quantification of nortriptyline and its active metabolites E- and Z-10-hydroxynortriptyline in human plasma. *Bioanalysis* 2010;2:1553-60.

Borg JJ, Tanti A, Kouvelas D, Lungu C, Pirozynski M, Serracino-Inglott A, et al. European Union pharmacovigilance capabilities: potential for the new legislation. *Ther Adv Drug Saf* 2015;6(4):120-40.

Borobia AM, Dapia I, Tong HY, Arias P, Muñoz M, Tenorio J, et al. Clinical implementation of pharmacogenetic testing in a hospital of the Spanish National Health System: Strategy and experience over 3 years. *Clin Transl Sci* 2018;11(2):189–99.

Boyle J, Eriksson ME, Gribble L, Gouni R, Johnsen S, Coppini DV, et al. Randomized, placebo-controlled comparison of amitriptyline, duloxetine, and pregabalin in patients with chronic diabetic peripheral neuropathic pain: impact on pain, polysomnographic sleep, daytime functioning, and quality of life. *Diabetes Care* 2012;35(12):2451-8.

Breaud AR, Harlan R, Di Bussolo JM, McMillin GA, Clarke W. A rapid and fully-automated method for the quantitation of tricyclic antidepressants in serum using turbulent-flow liquid chromatography-tandem mass spectrometry. *Clin Chim Acta* 2010;411:825-32.

Breyer-Pfaff U. The metabolic fate of amitriptyline, nortriptyline and amitriptylinoxide in man. *Drug Metab Rev* 2004;36:723-46.

Bryant SG, Fisher S, Kluge RM. Long-term versus short-term amitriptyline side effects as measured by a postmarketing surveillance system. *J Clin Psychopharmacol* 1987;7(2):78-82.

Bryson HM, Wilde MI. Amitriptyline. A review of its pharmacological properties and therapeutic use in chronic pain states. *Drugs Aging* 1996;8(6):459-76.

Bulla A, De Witt B, Ammerlaan W, Betsou F, Lescuyer P. Blood DNA yield but not integrity or methylation is impacted after long-term storage. *Biopreserv Biobank* 2016;14(1):29-38.

Burch JE, Hullin RP. Amitriptyline pharmacokinetics: A crossover study with single doses of amitriptyline and nortriptyline. *Psychopharmacology* 1981;74:35-42.

Calker D, Serchov T, Normann C, Biber K. Recent insights into antidepressant therapy: Distinct pathways and potential common mechanisms in the treatment of depressive syndromes. *Neurosci Biobehav Rev* 2018;88:63-72.

Cao Z, Kaleta E, WangP. Simultaneous quantitation of 78 drugs and metabolites in urine with a dilute-and-shoot LC-MS-MS assay. *J Anal Toxicol* 2015;39:335-46.

Cappelleri JC, Bienen EJ, Koduru V, Sadosky A. Measurement properties of painDETECT by average pain severity. *Clinicoecon Outcomes Res* 2014;6:497-504.

Caraballo PJ, Hodge LS, Bielinski SJ, Stewart AK, Farrugia G, Schultz CG, et al. Multidisciplinary model to implement pharmacogenomics at the point of care. *Genet Med* 2017;19(4):421-9.

Carrier F, Banayan D, Boley R, Karnik N. Ethical challenges in developing drugs for psychiatric disorders. *Prog Neurobiol* 2017;152:58-69.

Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington HL, et al. Influence of life stress on depression: Moderation by a polymorphism in the 5-HTT gene. *Science* 2003;301(5631):386-9.

Castro VM, Clements CC, Murphy SN, Gainer VS, Fava M, Weilburg JB, et al. QT interval and antidepressant use: a cross sectional study of electronic health records. *BMJ* 2013;346:f288.

Caudle KE, Dunnenberger HM, Freimuth RR, Peterson JF, Burlison JD, Whirl-Carrillo M, et al. Standardizing terms for clinical pharmacogenetic test results: consensus terms from the Clinical Pharmacogenetics Implementation Consortium (CPIC). *Genet Med* 2017;19(2):215-23.

Cavallari LH, Van Driest SL, Prows CA, Bishop JR, Limdi NA, Pratt VM, et al. Multi-site investigation of strategies for the clinical implementation of CYP2D6 genotyping to guide drug prescribing. *Genet Med* 2019. doi:10.1038/s41436-019-0484-3.

Ceskova E, Silhan P. Novel treatment options in depression and psychosis. *Neuropsychiatr Dis Treat* 2018;14:741–7.

Chae JW, Baek IH, An JH, Kim EJ, Kwon KI. Quantitative determination of amitriptyline and its metabolite in rat plasma by liquid chromatography-tandem mass spectrometry. *Bull Korean Chem Soc* 2012;33:2163-7.

Chambers EE, Woodcock MJ, Wheaton JP, Pekol TM, Diehl DM. Systematic development of an UPLC-MS/MS method for the determination of tricyclic antidepressants in human urine. *J Pharm Biomed Anal* 2014;88:660-5.

Chang MS, El-Shourbagy T. Throughput improvement of bioanalytical LC/MS/MS by sharing detector between HPLC systems. In: Wang PW, editor. High throughput analysis in the pharmaceutical industry. Boca Raton: CRC Press, Taylor & Francis Group;2009. p.119-140.

Chaudhary R, Singh B, Kumar M, Gakhar SK, Saini AK, Parmar VS, et al. Role of single nucleotide polymorphisms in pharmacogenomics and their association with human diseases. *Drug Metab Rev* 2015;47(3):281-90.

Cheung KC, Nijenhuis M, Crommentuijn–van Rhenen M, Holsappel I. Evidence-based pharmacogenetic guidelines in the Netherlands [Online]. Presented at FIP Congress; Stockholm; 2017 May [accessed 2019 Jul 27]. Available from: https://www.fip.org/www/?page=abstracts&action=generatePdf&item=18617.

Chogle A, Saps M. Electrocardiograms changes in children with functional gastrointestinal disorders on low dose amitriptyline. *World J Gastroenterol* 2014;20(32):11321-5.

Cipriani A, Furukawa TA, Salanti G, Chaimani A, Atkinson LZ, Ogawa Y, et al. Comparative efficacy and acceptability of 21 antidepressant drugs for the acute treatment of adults with major depressive disorder: a systematic review and network meta-analysis. *Lancet* 2018;391:1357–66.

Cleare A, Pariante CM, Young AH, Anderson IM, Christmas D, Cowen PJ, et al. Evidence-based guidelines for treating depressive disorders with antidepressants: A revision of the 2008 British Association for Psychopharmacology guidelines. *J Psychopharmacol* 2015;29(5):459–525.

Conrado DJ, Rogers HL, Zineh I, Pacanowski MA. Consistency of drug-drug and gene-drug interaction information in US FDA-approved drug labels. *Pharmacogenomics* 2013;14(2):215-23.

Cooper M, Eyre O, Doherty J, Jones RB. Gaining approvals for mental health research in the NHS. *B J Psych Advances* 2016;22:64-72.

Cottingham C, Percival S, Birky T, Wang Q. Tricyclic antidepressants exhibit variable pharmacological profiles at the α_{2A}adrenergic receptor. *Biochem Biophys Res Commun* 2014;451(3):461–6.

Coulter C, Taruc M, Tuyay J, Moore C. Antidepressant drugs in oral fluid using liquid chromatography-tandem mass spectrometry. *J Anal Toxicol* 2010;(34):64-72.

Crews KR, Gaedigk A, Dunnenberger HM, Klein TE, Shen DD, Callaghan JT, et al. Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines for codeine therapy in the context of cytochrome P450 2D6 (CYP2D6) genotype. *Clin Pharmacol Ther* 2012;91(2):321-6.

Cross-Disorder Group of the Psychiatric Genomics Consortium. Genetic relationship between five psychiatric disorders estimated from genome-wide SNPs. *Nat Genet* 2013;45(9):984–94.

da Cunha RJ, Barrucand L, Verçosa N. A study on electrocardiographic changes secondary to the use of tricyclic antidepressants in patients with chronic pain. *Rev Bras Anestesiol* 2009;59(1):46-55.

Dahl ML, Bertilsson L, Nordin C. Steady-state plasma levels of nortriptyline and its 10-hydroxy metabolite: relationship to the CYP2D6 genotype. *Psychopharmacology (Berl)* 1996;123(4):315-9.

Daimon CM, Cong W, Cai H, Wang R, Maudsley SR, Martin B et al. Neurometabolic effects of long-term amitriptyline administration in BDNF heterozygous knockout mice [Online]. Presented at The Endocrine Society's 95th Annual Meeting and Expo; San Francisco; 2013 Jun [accessed 2019 Jul 27]. Available from: https://www.endocrine.org/meetings/endo-annual-meetings/abstract-details?id=5109.

Dawes M, Aloise MN, Sidney Ang J, Cullis P, Dawes D, Fraser R, et al. Introducing pharmacogenetic testing with clinical decision support into primary care: a feasibility study. *CMAJ Open* 2016;4(3):E528–E534. doi:10.9778/cmajo.20150070.

Dawling S, Ford S, Rangedara DC, Lewis RR. Amitriptyline dosage prediction in elderly patients from plasma concentration at 24 hours after a single 100mg dose. *Clin Pharmacokinet* 1984;9:261-6.

Dawling S. Is there a practical alternative to therapeutic drug monitoring therapy with tricyclic antidepressants? *Clin Chem* 1988;34(5):841-7.

de Castro A, Concheiro M, Quintela O, Cruz A, Lo´ pez-Rivadulla M. LC–MS/MS method for the determination of nine antidepressants and some of their main metabolites in oral fluid and plasma - Study of correlation between venlafaxine concentrations in both matrices. *J Pharm Biomed Anal* 2008;48:183–93.

De Grandis G, Halgunset V. Conceptual and terminological confusion around personalised medicine: a coping strategy. *BMC Med Ethics* 2016;17(1):43.

de Leon J. Phenoconversion and therapeutic drug monitoring. *Br J Clin Pharmacol* 2015;80(4):777-8.

de Leon J, Susce MT, Johnson M, Hardin M, Maw L, Shao A, et al. DNA microarray technology in the clinical environment: the AmpliChip CYP450 test for CYP2D6 and CYP2C19 genotyping. *CNS Spectr* 2009;14(1):19-34.

Dean L. Amitriptyline therapy and CYP2D6 and CYP2C19 genotype. In: Pratt V, McLeod H, Dean L, Malheiro A, Rubinstein W, editors. Medical Genetics Summaries [Online]. Bethesda (MD): NCBI (US); 2017 [accessed 2019 Jul 27]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK425165/.

Del Tredici AL, Malhotra A, Dedek M, Espin F, Roach D, Zhu GD, et al. Frequency of CYP2D6 alleles including structural variants in the United States. *Front Pharmacol* 2018;9:305. doi:10.3389/fphar.2018.00305.

Dell'Aquila C. Separation of tricyclic antidepressants by capillary zone electrophoresis with N,N,N,N-tetramethyl-1,3-butanediamine (TMBD) as an effective electrolyte additive. *J Pharm Biomed Anal* 2002;30:341-50.

Dharmshaktu P, Tayal V, Kalra BS. Efficacy of antidepressants as analgesics: a review. *J Clin Pharmacol* 2012;52(1):6-17.

Donoghue J, Hylan TR. Antidepressant use in clinical practice: efficacy v. effectiveness. *Br J Psychiatry* 2001;179(Suppl 42):s9-s17.

Dove ES, Özdemir V. All the post-genomic world is a stage: the actors and narrators required for translating pharmacogenomics into public health. *Per Med* 2013;10(3):213–6.

Dressler LG, Bell GC, Abernathy PM, Ruch K, Denslow S. Implementing pharmacogenetic testing in rural primary care practices: a pilot feasibility study. *Pharmacogenomics* 2019;20(6):433-46.

Edelbroek PM, Zitman FG, Schreuder JN, Rooymans HGM, de Wolff FA. Amitriptyline metabolism in relation to antidepressive effect. *Clin Pharmacol Ther* 1984;35(4):467-73.

Ehmann F, Caneva L, Papaluca M. European Medicines Agency initiatives and perspectives on pharmacogenomics. *Br J Clin Pharmacol* 2014;77(4):612-7.

Ehmann F, Caneva L, Prasad K, Paulmichl M, Maliepaard M, Llerena A, et al. Pharmacogenomic information in drug labels: European Medicines Agency perspective. *Pharmacogenomics J* 2015;15(3):201-10.

Eikelenboom-Schieveld SJM, Fogleman JC. Letter regarding: Ekhart C, Matic M, Kant A, van Puijenbroek E, Schaik RV - CYP450 genotype and aggressive behavior on selective serotonin reuptake inhibitors. *Pharmacogenomics* 2018;19(14). doi:10.2217/pgs-2018-0203.

ElHoussini OM, Zawilla NH. Chromatographic methods for the determination of fluphenazine, nortriptyline and its impurity amitriptyline in bulk and pharmaceutical formulations. *J Anal Chem* 2014;69(12):1187–92.

Espinosa S, Bosch E, Rosés M. Retention of ionizable compounds in high-performance liquid chromatography 14. Acid-base pK values in acetonitrile-water mobile phases. *J Chromatogr A* 2002;964:55-66.

European Medicines Agency. Annual Report 2017 [Online]. UK; London: 2017 [accessed 2018 Dec 21]. Available from: https://www.ema.europa.eu/en/documents/annual-report/2017-annual-report-european-medicines-agency_en.pdf.

Evans SJ, Waller PC, Davis S. Use of proportional reporting ratios (PRRs) for signal generation from spontaneous adverse drug reaction reports. *Pharmacoepidemiol Drug Saf* 2001;10(6):483-6.

Fan M, Bousman CA. Commercial pharmacogenetic tests in psychiatry: Do they facilitate the implementation of pharmacogenetic dosing guidelines? *Pharmacopsychiatry* 2019;52:e1–e10. doi:10.1055/a-0863-4692.

Fanoe S, Kristensen D, Fink-Jensen A, Jensen HK, Toft E, Nielsen J, et al. Risk of arrhythmia induced by psychotropic medications: a proposal for clinical management. *Eur Heart J* 2014;35(20):1306-15.

Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, et al. Pharmacotherapy for neuropathic pain in adults: systematic review, meta-analysis and updated NeuPSIG recommendations. *Lancet Neurol* 2015;14(2):162–73.

Flint J, Kendler KS. The Genetics of Major Depression. *Neuron* 2014;81(3):484-503.

Formea CM, Nicholson WT, McCullough KB, Berg KD, Berg ML, Cunningham JL, et al. Development and evaluation of a pharmacogenomics educational program for pharmacists. *Am J Pharm Educ* 2013;77(1):10. doi:10.5688/ajpe77110.

Freynhagen R, Baron R, Gockel U, Tölle TR. painDETECT: a new screening questionnaire to identify neuropathic components in patients with back pain. *Curr Med Res Opin* 2006; 22(10):1911-20.

Freynhagen R, Tölle TR, Gockel U, Baron R. The painDETECT project - far more than a screening tool on neuropathic pain. *Curr Med Res Opin* 2016;32(6):1033-57.

Funai Y, Funao T, Ikenaga K, Takahashi R, Hase I, Nishikawa K. Use of tricyclic antidepressants as analgesic adjuvants results in non-hazardous prolongation of the QTc interval. *Osaka City Med J* 2014;60(1):11-9.

Gaedigk A, Dinh JC, Jeong H, Prasad B, Leeder JS. Ten years' experience with the CYP2D6 activity score: A perspective on future investigations to improve clinical predictions for precision therapeutics. *J Pers Med* 2018;8(2):15. doi:10.3390/jpm8020015.

Gaedigk A, Sangkuhl K, Whirl-Carrillo M, Klein T, Leeder JS. Prediction of CYP2D6 phenotype from genotype across world populations. *Genet Med* 2017;19(1):69-76.

Galaon T, David V. The influence of mobile phase pH on the retention and selectivity of related basic compounds in reversed-phase liquid chromatography. *Rev Roum Chim* 2012;57:131-40.

García-González J, Tansey KE, Hauser J, Henigsberg N, Maier W, Mors O, et al. Pharmacogenetics of antidepressant response: A polygenic approach. *Prog Neuropsychopharmacol Biol Psychiatry* 2017;75:128-34.

Gautam S, Jain A, Gautam M, Vahia VN, Grover S. Clinical Practice Guidelines for the management of depression. *Indian J Psychiatry* 2017;59(Suppl 1):S34–S50.

Gefenas E, Cekanauskaite A, Tuzaite E, Dranseika V, Characiejus D. Does the "new philosophy" in predictive, preventive and personalised medicine require new ethics? *EPMA J* 2011;2(2):141–7.

Georgi K, Boos KS. Multidimensional on-line SPE for undisturbed LC-MS-MS analysis of basic drugs in biofluids. *Chromatographia* 2006;63:523-31.

Ghatak S, Muthukumaran RB, Nachimuthu SK. A simple method of genomic DNA extraction from human samples for PCR-RFLP analysis. *J Biomol Tech* 2013;24(4):224–31.

Giller E Jr, Bialos D, Harkness L, Jatlow P, Waldo M. Long-term amitriptyline in chronic depression. *Hillside J Clin Psychiatry* 1985;7(1):16-33.

Gillman PK. Tricyclic antidepressant pharmacology and therapeutic drug interactions updated. *Br J Pharmacol* 2007;151(6):737–48.

Goeringer KE, McIntyre IM, Drummer OH. LC-MS analysis of serotonergic drugs. *J Anal Toxicol* 2003;27(1):30-5.

Goldstein JA, de Morais SM. Biochemistry and molecular biology of the human CYP2C subfamily. *Pharmacogenetics* 1994;4:285–99.

Gonzalez FJ, Skoda RC, Kimura S, Umeno M, Zanger UM, Nebert DW, et al. Characterization of the common genetic defect in humans deficient in debrisoquine metabolism. *Nature* 1988;331:442–6.

Goodnick PJ, Jerry J, Parra F. Psychotropic drugs and the ECG: focus on the QTc interval. *Expert Opin Pharmacother* 2002;3(5):479-98.

Graff-Radford SB, Shaw LR, Naliboff BN. Amitriptyline and fluphenazine in the treatment of postherpetic neuralgia. *Clinic J Pain* 2000;16(3):188-92.

Gupta AK, Thakur RK. Wide QRS complex tachycardias. *Med Clin North Am* 2001;85(2):245-66.

Gupta SK, Shah JC, Hwang SS. Pharmacokinetic and pharmacodynamic characterization of OROS® and immediate-release amitriptyline. *Br J Clin Pharmacol* 1999;48(1):71–8.

Gureje O, Von Korff M, Simon GE, Gater R. Persistent pain and wellbeing: a World Health Organization study in primary care. *JAMA* 1998;280(2):147–51.

Halling J, Weihe P, Brosen K. The CYP2D6 polymorphism in relation to the metabolism of amitriptyline and nortriptyline in the Faroese population. *Br J Clin Pharmacol* 2008;65(1):134-8.

Hamilton M. A rating scale for depression. *J Neurol Neurosurg Psychiatry* 1960;23:56-62.

Hamilton M. Development of a rating scale for primary depressive illness. *Br J Soc Clin Psychol* 1967;6(4):278-96.

Hamilton TC, Norton J, Poyser RH, Thormählen D. Comparison of some effects of paroxetine with amitriptyline on the cardiovascular system in animals. *Arzneimittelforschung* 1986;36(3):460-3.

Hansen SH, Pedersen-Bjergaard S, Rasmussen K. Analysis of Drugs in Biological Fluids. In: Introduction to pharmaceutical chemical analysis. West Sussex: John Wiley & Sons, Ltd; 2012. p. 299-301,466-68.

Hansen TV, Simonsen MK, Nielsen FC, Hundrup YA. Collection of blood, saliva, and buccal cell samples in a pilot study on the Danish nurse cohort: comparison of the response rate and quality of genomic DNA. *Cancer Epidemiol Biomarkers Prev* 2007;16:2072–6.

Harper AR, Topol EJ. Pharmacogenomics in clinical practice and drug development. *Nat Biotechnol* 2012;30(11):1117-24.

He Y, Hoskins JM, McLeod HL. Copy Number Variants in pharmacogenetic genes. *Trends Mol Med* 2011;17(5):244–51.

Hicks JK, Sangkuhl K, Swen JJ, Ellingrod VL, Müller DJ, Shimoda K et al. Clinical pharmacogenetics implementation consortium guideline (CPIC) for CYP2D6 and CYP2C19 genotypes and dosing of tricyclic antidepressants: 2016 update. *Clin Pharmacol Ther* 2017;102(1):37-44.

Hicks JK, Sangkuhl K, Swen JJ, Ellingrod VL, Müller DJ, Shimoda K et al. Clinical pharmacogenetics implementation consortium guideline (CPIC) for CYP2D6 and CYP2C19 genotypes and dosing of tricyclic antidepressants: 2016 update. *Clin Pharmacol Ther* 2017;102(Suppl):S1-S52.

Hicks JK, Swen JJ, Thorn CF, Sangkuhl K, Kharasch ED, Ellingrod VL, et al. Clinical Pharmacogenetics Implementation Consortium guideline for CYP2D6 and CYP2C19 genotypes and dosing of tricyclic antidepressants. *Clin Pharmacol Ther* 2013;93(5):402-8.

Hiemke C, Bergemann N, Clement HW, Conca A, Deckert J, Domschke K, et al. Consensus guidelines for therapeutic drug monitoring in neuropsychopharmacology: Update 2017. *Pharmacopsychiatry* 2018;51:9–62.

Hire RC, Kinage PJ, Gaikwad NN. Causality assessment in pharmacovigilance: a step towards quality care. *Sch J App Med Sci* 2013;1(5):386-92.

Hodgson K. Biomarkers of antidepressant treatment outcomes. PhD [Thesis]. London: King's College; 2014. Available from: King's Research Portal.

Horgan D, Jansen M, Leyens L, Lal JA, Sudbrak R, Hackenitz E, et al. An index of barriers for the implementation of personalised medicine and pharmacogenomics in Europe. *Public Health Genomics* 2014;17(5-6):287-98.

Hornberger J, Li Q, Quinn B. Cost-Effectiveness of combinatorial pharmacogenomic testing for treatment-resistant major depressive disorder patients. *Am J Manag Care* 2015;21(6):e357-e365.

ICH. Genomic Sampling and Management of Genomic Data. ICH Final Business Plan E18 [Online]. ICH; 2015 [accessed 2018 Dec 21]. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E18/E18EWG_BusinessPlan_Final_2015_1005.pdf.

ICH. Definitions for genomic biomarkers, pharmacogenomics, pharmacogenetics, genomic data and sample coding categories. ICH Harmonised Tripartite Guideline E15 [Online]. ICH; 2007 [accessed 2018 Dec 21]. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E15/Step4/E15_Guideline.pdf.

ICH. Genomic Biomarkers Related to Drug Response: Context, Structure and Format of Qualification Submissions. ICH Harmonised Tripartite Guideline E16 [Online]. ICH; 2010 [accessed 2018 Dec 21]. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E16/Step4/E16_Step_4.pdf.

ICH. Guideline on genomic sampling and management of genomic data. ICH Harmonised Guideline E18 [Online]. ICH; 2017 [accessed 2019 Jul 27]. Available from: https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E18/E18EWG_Step_4_Guideline_2017_0803.pdf.

ICH. The clinical evaluation of QT/QTc interval prolongation and proarrhythmic potential for nonantiarrhythmic drugs. ICH Harmonised Guideline E14 [Online]. ICH; 2005 [accessed 2018 Dec 21]. Available from: https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E14/E14_Guideline.pdf.

International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. *Nature* 2004;431:931–45.

Issa AM. Ethical perspectives on pharmacogenomic profiling in the drug development process. *Nat Rev Drug Discov* 2002;1(4):300-8.

Jackson KC, St. Onge EL. Antidepressant pharmacotherapy: considerations for the pain clinician. *Pain Pract* 2003;3(2):135–43.

Jain KK. Applications of AmpliChip CYP450. *Mol Diagn* 2005;9(3):119-27.

Janakiraman R, Hamilton L, Wan A. Unravelling the efficacy of antidepressants as analgesics. *Aust Fam Physician* 2016;45(3):113-7.

Jang SW, Liu X, Chan CB, Weinshenker D, Hall RA, Xiao G et al. The antidepressant amitriptyline is a TrkA and TrkB receptor agonist that promotes TrkA/TrkB heterodimerization and has potent neurotrophic activity. *Chem Biol* 2009;16(6):644–56.

Japan Pharmaceutical Manufacturers Association. Pharmaceutical Administration and Regulations in Japan. Regulatory Information Task Force [Online]. JPMA; 2017 [accessed 2018 Dec 21]. Available from: http://www.jpma.or.jp/english/parj/pdf/2017.pdf.

Jefferson JW. Clinical and pharmacologic perspectives on the treatment of major depressive disorder. *CNS Spectr* 2011;16(9):191-203.

Jensen TS, Baron R, Haanpää M, Kalso E, Loeser JD, Rice AS, et al. A new definition of neuropathic pain. *Pain* 2011;152(10):2204–5.

Jornil J, Linnet K. Roles of polymorphic enzymes CYP2D6 and CYP2C19 for in vitro metabolism of amitriptyline at therapeutic and toxic levels. *Forensic Toxicol* 2009;27:12–20.

Kamble SV, Motlekar SA, D'souza LL, Kudrigikar VN, Rao SE. Low doses of amitriptyline, pregabalin, and gabapentin are preferred for management of neuropathic pain in India: is there a need for revisiting dosing recommendations? *Korean J Pain* 2017;30(3):183-91.

Katz MM, Koslowa SH, Maasa JW, Frazera A, Kocsisa J, Secundaa S et al. Identifying the specific clinical actions of amitriptyline: interrelationships of behaviour, affect and plasma levels in depression. *Psychol Med* 1991;21(3):599-611.

Khatoon A, Setapar SHM, Ahmad A, Nian-Yian L. Analytical methodologies for determination of amitriptyline and its metabolite nortriptyline: A review. *Der Pharma Chemica* 2013;5(3):79-85.

Kirkland JJ, van Straten MA, Claessens HA. High pH mobile phase effects on silica-based reversed-phase high-performance liquid chromatographic columns. *J Chromatogr A* 1995;691:3-19.

Kishore Kumar H, Ravindranath LK, Jaya Veera KN, Kishore Kumar K, Ramachandra Reddy Y, Jagadeesh B, et al. Development and validation of a highly sensitive LC-MS/MS method for simultaneous quantitation of nortriptyline and 10-hydroxynortriptyline in human plasma: application to a human pharmacokinetic study. *Biomed Chromatogr* 2010;24:1113-9.

Kiss Á, Menus Á, Tóth K, Déri M, Sirok D, Gabri E, et al. Phenoconversion of CYP2D6 by inhibitors modifies aripiprazole exposure. *Eur Arch Psychiatry Clin Neurosci* 2019. doi:10.1007/s00406-018-0975-2.

Klieber M, Oberacher H, Hofstaetter S, Beer B, Neururer M, Amann A, et al. CYP2C19 Phenoconversion by routinely prescribed proton pump inhibitors omeprazole and esomeprazole: Clinical implications for personalized medicine. *J Pharmacol Exp Ther* 2015;354(3):426-30.

Kopsky DJ, Hesselink JM. High doses of topical amitriptyline in neuropathic pain: two cases and literature review. *Pain Pract* 2012;12(2):148-53.

Kousi M, Katsanis N. Genetic modifiers and oligogenic inheritance. *Cold Spring Harb Perspect Med* 2015;5:a017145. doi:10.1101/cshperspect.a017145.

Krynetski EY, Schuetz JD, Galpin AJ, Pui CH, Relling MV, Evans WE. A single-point mutation leading to loss of catalytic activity in human thiopurine S-methyltransferase. *Proc Natl Acad Sci USA* 1995;92:949–53.

Kuhn R. The treatment of depressive states with G 22355 (imipramine hydrochloride). *Am J Psychiatry* 1958;115:459–64.

Kwok PY, Chen X. Detection of single nucleotide polymorphisms. *Curr Issues Mol Biol* 2003;5(2):43-60.

La Russa R, Fineschi V, Di Sanzo M, Gatto V, Santurro A, Martini G, et al. Personalized medicine and adverse drug reactions: the experience of an Italian teaching hospital. *Curr Pharm Biotechnol* 2017;18(3):274-81.

Lakhan SE, Vieira K, Hamlat E. Biomarkers in psychiatry: drawbacks and potential for misuse. *Int Arch Med* 2010;3:1. doi:10.1186/1755-7682-3-1.

Langaee T, Hamadeh I, Chapman AB, Gums JG, Johnson JA. A novel simple method for determining CYP2D6 gene copy number and identifying allele(s) with duplication/multiplication. PLoS ONE 2015;10(1):e0113808. doi:10.1371/journal.pone.0113808.

Larsen JB, Rasmussen JB. Pharmacogenetic testing revisited: 5′ nuclease real-time polymerase chain reaction test panels for genotyping CYP2D6 and CYP2C19. *Pharmgenomics Pers Med* 2017;10:115–28.

Lauschke VM, Ingelman-Sundberg M. The importance of patient-specific factors for hepatic drug response and toxicity. *Int J Mol Sci* 2016;17(10):1714. doi:10.3390/ijms17101714.

Lawson K. A brief review of the pharmacology of amitriptyline and clinical outcomes in treating fibromyalgia. *Biomedicines* 2017;5(2):24. doi:10.3390/biomedicines5020024.

Lazaridis KN. Improving therapeutic odyssey: Preemptive pharmacogenomics utility in patient care. *Clin Pharmacol Ther* 2017;101(1):39-41.

Leijon G, Boivie J. Central post-stroke pain - a controlled trial of amitriptyline and carbamazepine. *Pain* 1989;36(1):27-36.

Levy F, Einfeld S, Looi J. Combined pharmacotherapy or polypharmacy? *J Paediatr Child Health* 1996;32:265-6.

Lieberman JA, Cooper TB, Suckow RF, Steinberg H, Borenstein M, Brenner R, et al. Tricyclic antidepressant and metabolite levels in chronic renal failure. *Clin Pharmacol Ther* 1985;37(3):301-7.

Liedgens H, Obradovic M, De Courcy J, Holbrook T, Jakubanis R. A burden of illness study for neuropathic pain in Europe. *CEOR* 2016;8:113-26.

Linden R, Venzon Antunes M, Ziulkoski AL, Wingert M, Tonello P, Tzvetkov M, et al. Determination of amitriptyline and its main metabolites in human plasma samples using HPLC-DAD: Application to the determination of metabolic ratios after single oral dose of amitriptyline. *J Braz Chem Soc* 2008;19(1):35-41.

Linder MW, Keck PE. Standards of laboratory practice: antidepressant drug monitoring. National Academy of Clinical Biochemistry. *Clin Chem* 1998;44(5):1073-84.

Lindor NM, Thibodeau SN, Burke W. Whole-genome sequencing in healthy people. *Mayo Clin Proc* 2017;92(1):159-72.

Lingjaerde O, Ahlfors UG, Bech P, Dencker SJ, Elgen K. The UKU side effect rating scale. A new comprehensive rating scale for psychotropic drugs and a cross-sectional study of side effects in neuroleptic-treated patients. *Acta Psychiatr Scand Suppl* 1987;334:1-100.

Lionte C, Bologa C, Sorodoc L. Toxic and drug-induced changes of the electrocardiogram. In: Millis R, editor. Advances in Electrocardiograms - Clinical Applications. INTECH Open Access Publisher; 2012. p. 271-96.

Lisbeth P, Vincent H, Kristof M, Bernard S, Manuel M, Hugo N. Genotype and co-medication dependent CYP2D6 metabolic activity: effects on serum concentrations of aripiprazole, haloperidol, risperidone, paliperidone and zuclopenthixol. *Eur J Clin Pharmacol* 2016;72(2):175-84.

Liu WQ, Kanungo A, Toth C. Equivalency of tricyclic antidepressants in open-label neuropathic pain study. *Acta Neurol Scand* 2014;129(2):132-41.

LLerena A, Naranjo ME, Rodrigues-Soares F, Penas-LLedó EM, Fariñas H, Tarazona-Santos E. Interethnic variability of CYP2D6 alleles and of predicted and measured metabolic phenotypes across world populations. *Expert Opin Drug Metab Toxicol* 2014;10:1569–83.

Loan GM, Wafai ZA, Shagufta W. Variability in systemic pharmacokinetics of amitriptyline by blood pressure alterations in patients of depression: A PD based PK analysis model. *IJRDPL* 2012;1(4):193-202.

Lozupone M, Panza F, Stella E, La Montagna M, Bisceglia P, Miscio G, et al. Pharmacogenetics of neurological and psychiatric diseases at older age: has the time come? *Expert Opin Drug Metab Toxicol* 2016;13(3):259-77.

Luo S, Michler K, Johnston P, Macfarlane PW. A comparison of commonly used QT correction formulae: the effect of heart rate on the QTc of normal ECGs. *J Electrocardiol* 2004;37(Suppl):81-90.

MacKichan JJ, McGory R. Interpretation of serum drug concentrations. In: Lee M, editor. Basic Skills in Interpreting Laboratory Data. 4th ed. Bethesda MD: ASHP; 2009. p. 109-11.

Malhotra AK, Zhang JP, Lencz T. Pharmacogenetics in psychiatry: translating research into clinical practice. *Mol Psychiatry* 2012;17(8):760-9.

Maliepaard M, Nofziger C, Papaluca M, Zineh I, Uyama Y, Prasad K, et al. Pharmacogenetics in the evaluation of new drugs: a multiregional regulatory perspective. *Nat Rev Drug Discov* 2013;12:103-15.

Manzo RH, Olivera ME, Amidon GL, Shah VP, Dressman JB, Barends DM. Biowaiver monographs for immediate release solid oral dosage forms: amitriptyline hydrochloride. *J Pharm Sci* 2006;95(5):966-73.

Marshall A. Getting the right drug into the right patient. *Nature Biotechnology* 1997;15:1249-52.

Maruf AA, Greenslade A, Arnold PD, Bousman C. Antidepressant pharmacogenetics in children and young adults: A systematic review. *J Affect Disord* 2019;254:98-108.

Maslej MM, Bolker BM, Russell MJ, Eaton K, Durisko Z, Hollon SD, et al. The mortality and myocardial effects of antidepressants are moderated by pre-existing cardiovascular disease: A meta-analysis. *Psychother Psychosom* 2017;86:268–82.

Max MB, Lynch SA, Muir J, Shoaf SE, Smoller B, Dubner R. Effects of desipramine, amitriptyline, and fluoxetine on pain in diabetic neuropathy. *N Engl J Med* 1992;326(19):1250-6.

Max MB, Schafer SC, Culnane M, Smoller B, Dubner R, Gracely RH. Amitriptyline, but not lorazepam, relieves postherpetic neuralgia. *Neurology* 1988;38(9):1427-32.

Mindham RH. Tricyclic antidepressants. In: Tyrer P, editor. Drugs in Psychiatric Practice. Cambridge: Cambridge University Press; 1982. p. 177-218.

Mishra S, Bhatnagar S, Goyal GN, Rana SP, Upadhya SP. A comparative efficacy of amitriptyline, gabapentin, and pregabalin in neuropathic cancer pain: a prospective randomized double-blind placebo-controlled study. *Am J Hosp Palliat Care* 2012;29(3):177-82.

Mitchell PB. Therapeutic drug monitoring of psychotropic medications. *Br J Clin Pharmacol* 2000;49:303-12.

Mizutani T. PM frequencies of major CYPs in Asians and Caucasians. *Drug Metab Rev* 2003;35:99–106.

Moaddeb J, Mills R, Haga SB. Community pharmacists' experience with pharmacogenetic testing. *J Am Pharm Assoc* 2015;55(6):587-94.

Moein MM, Beqqli AE, Abdel-Rehim M. Bioanalytical method development and validation: Critical concepts and strategies. *J Chromatogr B* 2017;1043:3-11.

Montgomery SA, Asberg M. A new depression scale designed to be sensitive to change. *Br J Psychiatry* 1979;134:382-89.

Moore RA, Derry S, Aldington D, Cole P, Wiffen PJ. Amitriptyline for neuropathic pain in adults [Online]. Cochrane Database of Systematic Reviews; 2015 [accessed 2019 Jul 27]. Available from: https://www.cochranelibrary.com/cdsr/doi/10.1002/14651858.CD008242.pub3/full.

Morita S, Shimoda K, Someya T, Yoshimura Y, Kamijima K, Kato N. Steady-state plasma levels of nortriptyline and its hydroxylated metabolites in Japanese patients: impact of CYP2D6 genotype on the hydroxylation of nortriptyline. *J Clin Psychopharmacol* 2000;20(2):141-9.

Morlock R, Braunstein GD. Pharmacoeconomics of genotyping-based treatment decisions in patients with chronic pain. *Pain Rep* 2017;2(5):e615. doi:10.1097/PR9.0000000000000615.

Mulot C, Stücker I, Clavel J, Beaune P, Loriot MA. Collection of human genomic DNA from buccal cells for genetics studies: comparison between cytobrush, mouthwash, and treated card. *J Biomed Biotechnol* 2005;3:291–6.

Nachimuthu S, Assar MD, Schussler JM. Drug-induced QT interval prolongation: mechanisms and clinical management. *Ther Adv Drug Saf* 2012;3(5):241-53.

Najafzadeh M, Garces JA, Maciel A. Economic evaluation of implementing a novel pharmacogenomic test (IDgenetix®) to guide treatment of patients with depression and/or anxiety. *Pharmacoeconomics* 2017;35(12):1297-310.

Nam Y, Lim CH, Lee HS, Chung SJ, Chung YH, Shin YK, et al. Single-dose, randomized, open-label, 2-way crossover study of the pharmacokinetics of amitriptyline hydrochloride 10- and 25-mg tablet in healthy male Korean volunteers. *Clini Ther* 2015;37(2):302-10.

Nassan M, Nicholson WT, Elliott MA, Rohrer Vitek CR, Black JL, Frye MA et al. Pharmacokinetic pharmacogenetic prescribing guidelines for antidepressants: A template for psychiatric precision medicine. *Mayo Clin Proc* 2016;91(7):897-907.

Nebert DW, Vesell ES. Advances in pharmacogenomics and individualized drug therapy: exciting challenges that lie ahead. *Eur J Pharmacol* 2004;500:267–80.

Nebert DW, Zhang G, Vesell ES. From human genetics and genomics to pharmacogenetics and pharmacogenomics: past lessons, future directions. *Drug Metab Rev* 2008;40(2):187–224.

Nederlof M, Stoker LJ, Egberts TC, Heerdink ER. Instructions for clinical and biomarker monitoring in the Summary of Product Characteristics (SmPC) for psychotropic drugs: Overview and applicability in clinical practice. *J Psychopharmacol* 2015;29(12):1248-54.

Nelson JC. Tricyclic and tetracyclic drugs. In: Schatzberg AF, Nemeroff CB, editors. The American Psychiatric Publishing Textbook of Psychopharmacology. 4th ed. Washington DC: American Psychiatric Publishing; 2009. p. 263-87.

Neue UD, Méndez A, Tran K, Diehl DM. pH and selectivity in RP-chromatography. In: Kromidas S, editor. HPLC Made to Measure: A Practical Handbook for Optimization. Weinheim: Wiley-VCH; 2006. p. 71-87.

Nofziger C, Papaluca M, Terzic A, Waldman S, Paulmichl M. Policies to aid the adoption of personalized medicine. *Nat Rev Drug Discov* 2014;13(3):159–60.

Noordam R, van den Berg ME, Niemeijer MN, Aarts N, Leening MJ, Deckers JW, et al. Assessing prolongation of the heart rate corrected QT interval in users of tricyclic antidepressants: advice to use Fridericia rather than Bazett's correction. *J Clin Psychopharmacol* 2015;35(3):260-5.

Nordin C, Bertilsson L. Active hydroxymetabolites of antidepressants. Emphasis on E-10-hydroxy-nortriptyline. *Clin Pharmacokinet* 1995;28:26–40.

Nurnberger JI, Berrettini W, Niculescu AB. Genetics of Psychiatric Disorders. In: Fatemi SH, Clayton PJ, editors. The Medical Basis of Psychiatry. 4th ed. New York: Springer; 2016. p. 553-600.

OECD. Regulatory authorities and reimbursement mechanisms. In: Pharmacogenetics – Opportunities and challenges for health innovation. Paris, France: Organisation for economic co-operation and development; 2009. p. 105-6.

Ohno Y, Kusama M, Hisaka A, Yanagihara Y, Suzuki H. Analysis of pharmacokinetic data provided in Japanese package inserts and interview forms focusing on urinary excretion of pharmacologically active species. *Yakugaku Zasshi* 2006;126(7):489-94.

Okayasu H, Ozeki Y, Fujii K, Takano Y, Saeki Y, Hori H, et al. Pharmacotherapeutic determinants for QTc interval prolongation in Japanese patients with mood disorder. *Pharmacopsychiatry* 2012;45(7):279-83.

Olesen OV, Linnet K. Metabolism of the tricyclic antidepressant amitriptyline by cDNA-expressed human cytochrome P450 enzymes. *Pharmacology* 1997;55(5):235-43.

Olivier C, Williams-Jones B. Pharmacogenomic technologies: a necessary "luxury" for better global public health? *Global Health* 2011;7:30. doi:10.1186/1744-8603-7-30.

Onali P, Dedoni S, Olianas MC. Direct agonist activity of tricyclic antidepressants at distinct opioid receptor subtypes. *J Pharmacol Exp Ther* 2010;332(1):255-65.

Ostad Haji E, Hiemke C, Pfuhlmann B. Therapeutic drug monitoring for antidepressant drug treatment. *Curr Pharm Des* 2012;18:5818-27.

Owen RP, Sangkuhl K, Klein TE, Altman RB. Cytochrome P450 2D6. *Pharmacogenet Genomics* 2009;19(7):559–62.

Ozdemir V, Suarez-Kurtz G, Stenne R, Somogyi AA, Someya T, Kayaalp SO, et al. Risk assessment and communication tools for genotype associations with multifactorial phenotypes: the concept of "edge effect" and cultivating an ethical bridge between omics innovations and society. *OMICS* 2009;13(1):43-61.

Paksu S, Duran L, Altuntas M, Zengin H, Salis O, Ozsevik S, et al. Amitriptyline overdose in emergency department of university hospital: Evaluation of 250 patients. *Hum Exp Toxicol* 2014;33(9):980-90.

Pandey S, Pandey P, Tiwari G, Tiwari R. Bioanalysis in drug discovery and development. *Pharm Methods* 2010;1(1):14–24.

Patrinos GP, Katsila T. Pharmacogenomics education and research at the Department of Pharmacy, University of Patras, Greece. *Pharmacogenomics* 2016;17(17):1865-72.

Peñas-Lledó EM, Trejo HD, Dorado P, Ortega A, Jung H, Alonso E, et al. CYP2D6 ultrarapid metabolism and early dropout from fluoxetine or amitriptyline monotherapy treatment in major depressive patients. *Mol Psychiatry* 2013;18(1):8-9.

Pereira LP, Köhler CA, Stubbs B, Miskowiak KW, Morris G, de Freitas BP, et al. Imaging genetics paradigms in depression research: Systematic review and meta-analysis. *Prog Neuropsychopharmacol Biol Psychiatry* 2018;86:102-13.

Pesce A, West C, West R, Latyshev S, Masters-Moore D, Friel P, et al. Analytical considerations when monitoring pain medications by LC-MS/MS. *J Anal Bioanal Tech* 2012;S5:003. doi:10.4172/2155-9872.S5-003.

Phillips KA, Veenstra DL, Oren E, Lee JK, Sadee W. Potential role of pharmacogenomics in reducing adverse drug reactions: a systematic review. *JAMA* 2001;286:2270–9.

Picard N, Boyer JC, Etienne-Grimaldi MC, Barin-Le Guellec C, Thomas F, Loriot MA, et al. Pharmacogenetics-based personalized therapy: Levels of evidence and recommendations from the French Network of Pharmacogenetics (RNPGx). *Therapie* 2017;72(2):185-92.

Pinto N, Dolan ME. Clinically relevant genetic variations in drug metabolizing enzymes. *Curr Drug Metab* 2011;12(5):487–97.

Polasek TM, Lin FP, Miners JO, Doogue MP. Perpetrators of pharmacokinetic drug-drug interactions arising from altered cytochrome P450 activity: a criteria-based assessment. *Br J Clin Pharmacol* 2011;71(5):727-36.

Pollock BG, Everett G, Perel JM. Comparative cardiotoxicity of nortriptyline and its isomeric 10-hydroxymetabolites. *Neuropsychopharmacology* 1992;6(1):1-10.

Pondman KM, van Schaik RHN, van der Weide J. Accurate determination of the CYP2D6 (*1/*4)xN genotype by quantitative PCR. *Drug Metab Pers Ther* 2018;33(1):33-9.

Prasad K. Role of regulatory agencies in translating pharmacogenetics to the clinics. *Clin Cases Miner Bone Metab* 2009;6(1):29-34.

Prasad K, Breckenridge A. Pharmacogenomics: a new clinical or regulatory paradigm? European experiences of pharmacogenomics in drug regulation and regulatory initiatives. *Drug Discov Today* 2011;16:867-72.

Pratt VM, Del Tredici AL, Hachad H, Ji Y, Kalman LV, Scott SA, et al. Recommendations for clinical CYP2C19 genotyping allele selection: A report of the association for molecular pathology. *J Mol Diagn* 2018;20(3):269-76.

Preskorn SH. Personalized medicine in psychiatry: concepts for bringing associated testing into clinical practice. *Mayo Clin Proc* 2016;91(7):827-9.

Preskorn SH, Kane CP, Lobello K, Nichols AI, Fayyad R, Buckley G, et al. Cytochrome P450 2D6 phenoconversion is common in patients being treated for depression: implications for personalized medicine. *J Clin Psychiatry* 2013;74(6):614-21.

Puri BK, Teasaden IH. Textbook of Psychiatry. London: Churchill Livingstone Elsevier; 2011. p. 28-30.

Pushpakom SP, Pirmohamed M. Pharmacogenetics of adverse drug reactions. In: Talbot J, Aronson JK, editors. Stephens' Detection and Evaluation of Adverse Drug Reactions: Principles and Practice. 6th ed. West Sussex UK: John Wiley & Sons; 2012. p. 139-41.

Qaseem A, Barry MJ, Kansagara D, Clinical Guidelines Committee of the American College of Physicians. Non-pharmacologic versus pharmacologic treatment of adult patients with major depressive disorder: A Clinical Practice Guideline from the American College of Physicians. *Ann Intern Med* 2016;164:350–9.

Ramos L. Critical overview of selected contemporary sample preparation techniques. *J Chromatogr A* 2012;1221:84-98.

Rantamäki T, Vesa L, Antila H, Di Lieto A, Tammela P, Schmitt A, et al. Antidepressant drugs transactivate TrkB neurotrophin receptors in the adult rodent brain independently of BDNF and monoamine transporter blockade. *PLoS One* 2011;6(6):e20567. doi:10.1371/journal.pone.0020567.

Rao G, Goyal A. An overview on analytical method development and validation by using HPLC. *The Pharmaceutical and Chemical Journal* 2016;3(2):280-9.

Reis-Pardal J, Rodrigues A, Rodrigues E, Fernandez-Llimos F. Comparing cytochrome P450 pharmacogenetic information available on United States drug labels and European Union Summaries of Product Characteristics. *Pharmacogenomics J* 2017;17(6):488-93.

Rethmeyer JA, Tan X, Manzardo A, Schroeder SR, Butler MG. Comparison of biological specimens and DNA collection methods for PCR amplification and microarray analysis. *Clin Chem Lab Med* 2013;51(5):79–83.

Richardson AJ, Narendran N, Guymer RH, Vu H, Baird PN. Blood storage at 4°C - factors involved in DNA yield and quality. *J Lab Clin Med* 2006;147(6):290-4.

Rico-Villademoros F, Slim M, Calandre EP. Amitriptyline for the treatment of fibromyalgia: a comprehensive review. *Expert Rev Neurother* 2015;15(10):1123-50.

Rikkert MG, Lauque S, Frölich L, Vellas B, Dekkers W. The practice of obtaining approval from medical research ethics committees: a comparison within 12 European countries for a descriptive study on acetylcholinesterase inhibitors in Alzheimer's dementia. *Eur J Neurol* 2005;12(3):212-7.

Rintala DH, Holmes SA, Courtade D, Fiess RN, Tastard LV, Loubser PG. Comparison of the effectiveness of amitriptyline and gabapentin on chronic neuropathic pain in persons with spinal cord injury. *Arch Phys Med Rehabil* 2007;88(12):1547-60.

Robinson DS, Nies A, Corcella J, Cooper TB, Spencer C, Keefover R. Cardiovascular effects of phenelzine and amitriptyline in depressed outpatients. *J Clin Psychiatry* 1982;43:8-15.

Rochester MP, Kane AM, Linnebur SA, Fixen DR. Evaluating the risk of QTc prolongation associated with antidepressant use in older adults: a review of the evidence. *Ther Adv Drug Saf* 2018; 9(6):297-308.

Roederer MW, Kuo GM, Kisor DF, Frye RF, Hoffman JM, Jenkins J, et al. Pharmacogenomics competencies in pharmacy practice: A blueprint for change. *J Am Pharm Assoc* 2017;57(1):120-5.

Roffman JL, Silverman BC, Stern TA. Diagnostic Rating Scales and Laboratory Tests. In: Stern TA, Fricchione GL, Cassem NH, Jellinek M, Rosenbaum JF, editors. Massachusetts General Hospital Handbook of General Hospital Psychiatry. 6th ed. Philadelphia: Saunders Elsevier; 2010. p. 61-71.

Rowbotham MC, Reisner LA, Davies PS, Fields HL. Treatment response in antidepressant-naïve postherpetic neuralgia patients: double-blind, randomized trial. *J Pain* 2005;6(11):741-6.

Rush AJ, Trivedi MH, Wisniewski SR, Nierenberg AA, Stewart JW, Warden D, et al. Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report. *Am J Psychiatry* 2006;163(11):1905-17.

Ryu S, Park S, Lee JH, Kim YR, Na HS, Lim HS, et al. A study on CYP2C19 and CYP2D6 polymorphic effects on pharmacokinetics and pharmacodynamics of amitriptyline in healthy Koreans. *Clin Transl Sci* 2017;10(2):93-101.

Sadée W, Dai Z. Pharmacogenetics/genomics and personalized medicine. *Hum Mol Genet* 2005;14(2):R207-14.

Sadosky A, Koduru V, Bienen EJ, Cappelleri JC. Characterizing individual painDETECT symptoms by average pain severity. *Clinicoecon Outcomes Res* 2016;8:361-6.

Samanidou VF, Nika MK, Papadoyannis IN. HPLC as a tool in medicinal chemistry for the monitoring of tricyclic antidepressants in biofluids. *Mini-Rev Med Chem* 2008;8:256-75.

Samwald M, Xu H, Blagec K, Empey PE, Malone DC, Ahmed SM, et al. Incidence of exposure of patients in the united states to multiple drugs for which pharmacogenomic guidelines are available. *PLoS One* 2016;11(10):e0164972. doi:10.1371/journal.pone.0164972.

Sansone RA, Sansone LA. Pain, pain, go away: Antidepressants and pain management. *Psychiatry* 2008;5(12):16–19.

Scantamburlo G, Tziolia K, Zopf M, Bernardinelli E, Soyal SM, Civello DA, et al. Allele drop out conferred by a frequent CYP2D6 genetic variation for commonly used CYP2D6*3 genotyping assays. *Cell Physiol Biochem* 2017;43(6):2297-309.

Schaffner K. The person and philosophy of science and medicine. *Int J Integr Care* 2010; 10(5):e017. doi:10.5334/ijic.487.

Schneider LS, Cooper TB, Severson JA, Zemplenyi T, Sloane RB. Electrocardiographic changes with nortriptyline and 10-hydroxynortriptyline in elderly depressed outpatients. *J Clin Psychopharmacol* 1988;8(6):402-8.

Schulz P, Dick P, Blaschke TF, Hollister L. Discrepancies between pharmacokinetic studies of amitriptyline. *Clin Pharmacokinet* 1985;10(3):257-68.

Shah RR, Gaedigk A, LLerena A, Eichelbaum M, Stingl J, Smith RL. CYP450 genotype and pharmacogenetic association studies: a critical appraisal. *Pharmacogenomics* 2016;17(3):259-75.

Shah RR, Shah DR. Personalized medicine: is it a pharmacogenetic mirage? *Br J Clin Pharmacol* 2012;74(4):698-721.

Shah RR, Smith RL. Addressing phenoconversion: the Achilles' heel of personalized medicine. *Br J Clin Pharmacol* 2015;79(2):222–40.

Shah RR, Smith RL. Inflammation-induced phenoconversion of polymorphic drug metabolizing enzymes: hypothesis with implications for personalized medicine. *Drug Metab Dispos* 2015;43(3):400-10.

Shah RR, Smith RL. Phenocopy and phenoconversion: do they complicate association studies? *Pharmacogenomics* 2012;13(9):981-4.

Shaw K, Amstutz U, Carleton BC. Using pharmacogenetics to understand adverse drug reactions in children. *Paediatr Child Health* 2011;16(9):537–8.

Shimazawa R, Ikeda M. Differences in pharmacogenomic biomarker information in package inserts from the United States, the United Kingdom and Japan. *J Clin Pharm Ther* 2013;38(6):468-75.

Shimoda K, Someya T, Yokono A, Morita S, Hirokane G, Takahashi S, et al. The impact of CYP2C19 and CYP2D6 genotypes on metabolism of amitriptyline in Japanese psychiatric patients. *J Clin Psychopharmacol* 2002;22(4):371–8.

Shimoda K, Yasuda S, Morita S, Shibasaki M, Someya T, Bertilsson L, et al. Significance of monitoring plasma levels of amitriptyline, and its hydroxylated and desmethylated metabolites in prediction of the clinical outcome of depressive state. *Psychiatry Clin Neurosci* 1997;51;35-41.

Sinyor M, Schaffer A, Levitt A. The sequenced treatment alternatives to relieve depression (STAR*D) trial: a review. *Can J Psychiatry* 2010;55(3):126-35.

Slattery J, Alvarez Y, Hidalgo A. Choosing thresholds for statistical signal detection with the proportional reporting ratio. *Drug Saf* 2013;36:687–92.

Snyder LH. Studies in human inheritance. IX: The inheritance of taste deficiency in man. *Ohio J Sci* 1932;32(5):436–40.

Spina E, de Leon J. Clinical applications of CYP genotyping in psychiatry. *J Neural Transm* 2015;122(1):5-28.

Steimer W, Zöpf K, von Amelunxen S, Pfeiffer H, Bachofer J, Popp J, et al. Allele-specific change of concentration and functional gene dose for the prediction of steady-state serum concentrations of amitriptyline and nortriptyline in CYP2C19 and CYP2D6 extensive and intermediate metabolizers. *Clin Chem* 2004;50(9):1623-33.

Steimer W, Zöpf K, von Amelunxen S, Pfeiffer H, Bachofer J, Popp J, et al. Amitriptyline or not, that is the question: pharmacogenetic testing of CYP2D6 and CYP2C19 identifies patients with low or high risk for side effects in amitriptyline therapy. *Clin Chem* 2005;51(2):376-85.

Stern S, Linker S, Vadodaria KC, Marchetto MC, Gage FH. Prediction of response to drug therapy in psychiatric disorders. *Open Biol* 2018;8(5). doi:10.1098/rsob.180031.

Storelli F, Desmeules J, Daali Y. Physiologically-based pharmacokinetic modeling for the prediction of CYP2D6-mediated gene-drug-drug interactions. *CPT Pharmacometrics Syst Pharmacol* 2019. doi:10.1002/psp4.12411.

Storelli F, Matthey A, Lenglet S, Thomas A, Desmeules J, Daali Y. Impact of CYP2D6 functional allelic variations on phenoconversion and drug-drug interactions. *Clin Pharmacol Ther* 2018;104(1):148-57.

Subirats X, Rosés M, Bosch E. On the effect of organic solvent composition on the pH of buffered HPLC mobile phases and the pKa of analytes - A review. *Sep Purif Rev* 2007;36:231–55.

Sugarman EA, Cullors A, Centeno J, Taylor D. Contribution of pharmacogenetic testing to modeled medication change recommendations in a long-term care population with polypharmacy. *Drugs Aging* 2016;33(12):929–36.

Swen JJ, Nijenhuis M, van Rhenen M, de Boer-Veger NJ, Buunk AM, Houwink EJF. Pharmacogenetic information in clinical guidelines: The European Perspective. *Clin Pharmacol Ther* 2018;103(5):795-801.

Swinfield CE, Graham EAM, Nuttall D, Maguire S, Kemp A, Rutty GN. The use of DNA stabilizing solution to enable room temperature storage and transportation of buccal and trace sample swabs. *Forensic Sci Int Genet Suppl Ser* 2009;2(1):183-4.

Sykiotis GP, Kalliolias GD, Papavassiliou AG. Pharmacogenetic principles in the Hippocratic writings. *J Clin Pharmacol* 2005;45(11):1218-20.

Tremblay J, Hamet P. Role of genomics on the path to personalized medicine. *Metabolism* 2013;62(Suppl 1):S2-5.

Trinkley KE, Page RL, Lien H, Yamanouye K, Tisdale JE. QT interval prolongation and the risk of torsades de pointes: essentials for clinicians. *Curr Med Res Opin* 2013;29(12):1719-26.

Uddin MN, Samanidou VF, Papadoyannis IN. Bio-sample preparation and analytical methods for the determination of tricyclic antidepressants. *Bioanalysis* 2011;3(1):97–118.

Uher R, Farmer A, Henigsberg N, Rietschel M, Mors O, Maier W, et al. Adverse reactions to antidepressants. *Br J Psychiatry* 2009;195(3):202-10.

Uher R, Farmer A, Maier W, Rietschel M, Hauser J, Marusic A, et al. Measuring depression: comparison and integration of three scales in the GENDEP study. *Psychol Med* 2008;38(2):289-300.

UK Pharmacogenetics Study Group. Policy issues in pharmacogenetics: A policy briefing from the UK Pharmacogenetics Study Group [Online]. UK Pharmacogenetics Study Group; 2006 [accessed 2019 Jul 27]. Available from: https://www.york.ac.uk/res/pgx/publications/PGxpolicyissues2006.pdf.

Upward JW, Edwards JG, Goldie A, Waller DG. Comparative effects of fluoxetine and amitriptyline on cardiac function. *Br J Clin Pharmac* 1988;26:399-402.

van der Wouden CH, Cambon-Thomsen A, Cecchin E, Cheung KC, Dávila-Fajardo CL, Deneer VH, et al. Implementing pharmacogenomics in Europe: Design and implementation strategy of the Ubiquitous Pharmacogenomics Consortium. *Clin Pharmacol Ther* 2017;101(3):341-58.

van Hecke O, Austin SK, Khan RA, Smith BH, Torrance N. Neuropathic pain in the general population: a systematic review of epidemiological studies. *Pain* 2014;155(4):654-62.

Vandenberk B, Vandael E, Robyns T, Vandenberghe J, Garweg C, Foulon V, et al. Which QT correction formulae to use for QT monitoring? *J Am Heart Assoc* 2016;5(6):e003264. doi:10.1161/JAHA.116.003264.

Vassy JL, Davis JK, Kirby C, Richardson IJ, Green RC, McGuire AL, et al. How primary care providers talk to patients about genome sequencing results: Risk, rationale, and recommendation. *J Gen Intern Med* 2018;33(6):877-85.

Vella J, Mifsud M, Sammut Bartolo N, Ferrito V, Serracino Inglott A, Azzopardi LM, et al. The combined effect of pH and acetonitrile composition on the separation of two lincosamide antibiotics. *Asian J Pharm Clin Res* 2014;7(5):96-100.

Venkatakrishnan K, Greenblatt DJ, von Moltke LL, Schmider J, Harmatz JS, Shader RI. Five distinct human cytochromes mediate amitriptyline N-demethylation in vitro: Dominance of CYP 2C19 and 3A4. *Clin Pharmacol* 1998;38:112-21.

Verbeurgt P, Mamiya T, Oesterheld J. How common are drug and gene interactions? Prevalence in a sample of 1143 patients with CYP2C9, CYP2C19 and CYP2D6 genotyping. *Pharmacogenomics* 2014;15(5):655-65.

Vogel F. Moderne probleme der humangenetik. *Ergeb Inn Med Kinderheilkd* 1959;12:52–125.

Vrethem M, Boivie J, Arnqvist H, Holmgren H, Lindström T, Thorell LH. A comparison of amitriptyline and maprotiline in the treatment of painful polyneuropathy in diabetics and nondiabetics. *Clinic J Pain* 1997;13(4):313-23.

Weiller E, Weiss C, Watling CP, Edge C, Hobart M, Eriksson H et al. Functioning outcomes with adjunctive treatments for major depressive disorder: a systematic review of randomized placebo-controlled studies. *Neuropsychiatr Dis Treat* 2018;14:103–15.

Weinshilboum RM, Wang L. Pharmacogenomics: Precision medicine and drug response. *Mayo Clin Proc* 2017;92(11):1711-22.

Wilke RA, Mareedu RK, Moore JH. The pathway less traveled: moving from candidate genes to candidate pathways in the analysis of genome-wide data from large scale pharmacogenetic association studies. *Curr Pharmacogenomics Person Med* 2008;6(3):150–59.

Wirth F, Zahra G, Xuereb RG, Barbara C, Fenech A, Azzopardi LM. Comparison of a rapid point-of-care and two laboratory-based CYP2C19*2 genotyping assays for personalisation of antiplatelet therapy. *Int J Clin Pharm* 2016:38(2):414-20.

Wray NR, Ripke S, Mattheisen M, Trzaskowski M, Byrne EM, Abdallaoui A, et al. Genome-wide association analyses identify 44 risk variants and refine the genetic architecture of major depression. *Nature Genet* 2018;50:668-81.

Wu AH. Drug metabolizing enzyme activities versus genetic variances for drug of clinical pharmacogenomic relevance. *Clin Proteomics* 2011;8(1):12.

Yap YG, Camm AJ. Drug induced QT prolongation and torsades de pointes. *Heart* 2003;89:1363–72.

Yekehtaz H, Farokhnia M, Akhondzadeh S. Cardiovascular considerations in antidepressant therapy: an evidence-based review. *J Tehran Heart Cent* 2013;8(4):169–76.

Young RC, Alexopoulos GS, Shamoian CA, Dhar AK, Kutt H. Heart failure associated with high plasma 10-hydroxynortriptyline levels. *Am J Psychiatry* 1984;141(3):432-3.

Zhou X, Chen C, Zhang F, Zhang Y, Feng Y, Ouyang H, et al. Metabolism and bioactivation of the tricyclic antidepressant amitriptyline in human liver microsomes and human urine. *Bioanalysis* 2016;8:1365-81.

Ziegler VE, Co BT, Biggs JT. Plasma nortriptyline levels and ECG findings. *Am J Psychiatry* 1977;134(4):441-3.

List of Publications and Abstracts

Research papers

I. Mifsud Buhagiar L, Sammut C, Chircop Y, Axisa K, Sammut Bartolo N, Vella Szijj J, Serracino Inglott A, LaFerla G. Practical LC–MS/MS method for the simultaneous quantification of amitriptyline, nortriptyline and their hydroxy-metabolites in human serum. *Biomed Chromatogr* 2019. doi:10.1002/bmc.4679.

II. Mifsud Buhagiar L, Casha M, Grech A, Micallef B, Borg JJ, Serracino Inglott A, LaFerla G. Safety implications of low-dose amitriptyline in neuropathic pain. *Pharm Front* 2019;1:e190003. doi:10.20900/pf20190003.

III. Mifsud Buhagiar L, Micallef B, Borg JJ, Vella H, Serracino Inglott A, LaFerla G. Regulatory sciences and translational pharmacogenetics: amitriptyline as a case in point. *Drug Metab Pers Ther* 2019;34(2). doi:10.1515/dmpt-2019-0005.

IV. Mifsud Buhagiar L, Scorpiniti M, Sammut Bartolo N, Vella Szijj J, Ferrito V, Serracino Inglott A, LaFerla G. Implications of mobile phase composition and pH on the chromatographic separation of amitriptyline and its metabolite nortriptyline. *Int J Pharm Pharm Sci* 2018;10(4):132-8. doi:10.22159/ijpps.2018v10i4.24817.

Presentations

i. Mifsud Buhagiar L, Wirth F, Serracino Inglott A. Pharmacogenetics as a tool for clinical pharmacists to promote precision medicine in a digitalised environment. Accepted as workshop for the 48th Symposium of the European Society of Clinical Pharmacy; 2019 Oct 23–25; Ljubljana, Slovenia.

ii. Mifsud Buhagiar L, Vella K, Grech A, Serracino Inglott A, LaFerla G. Multi-disciplinary research in psychiatry: the ethics of capacity. Poster presented at the Junior College Multi-Disciplinary Conference: Research, Practice and Collaboration; 2019 Sept 18-20; Msida, Malta.

iii. Mifsud Buhagiar L, Serracino Inglott A, LaFerla G. Empowering pharmacy students to participate in the clinical implementation of pharmacogenetics. Oral presentation delivered at the European Association of Faculties of Pharmacy Annual Conference; 2019 May 15–17; Kraków, Poland.

iv. Mifsud Buhagiar L, Sammut Bartolo N, Vella Szijj J, Ferrito V, Serracino Inglott A, LaFerla G. Analysis of amitriptyline and its metabolites. Poster presented at the 10th Malta Medical School Conference; 2018 Nov 29 – Dec 1; Malta.

v. Mifsud Buhagiar L, Flores G, Serracino Inglott A. Translating genomics science into personalized medicine: the regulatory aspect. Poster presented at the American College of Clinical Pharmacy Global Conference on Clinical Pharmacy; 2018 Oct 20–23; Seattle, Washington.

vi. Mifsud Buhagiar L, Serracino Inglott A, Laferla G. Pharmacogenetics as a tool for teaching precision medicine. Poster presentation at the European Association of Faculties of Pharmacy Annual Conference; 2018 May 16–18; Parma, Italy.

vii. Mifsud Buhagiar L, Grech A, Casha M, Serracino Inglott A, LaFerla G. The role of pharmacogenetics in rediscovering old drugs – spotlight on amitriptyline. Poster presented at the European Society of Pharmacogenomics and Personalised Therapy Fourth Conference; 2017 Oct 4–7; Catania, Italy.

viii. Mifsud Buhagiar L, Grech A, Serracino Inglott A, LaFerla G. Amitriptyline revisited - pharmacogenetic implications in antidepressant treatment. Poster presentation at the 77th International Pharmaceutical Federation World Congress of Pharmacy and Pharmaceutical Sciences; 2017 Sept 10–14; Seoul, South Korea.

ix. Mifsud Buhagiar L, Grech A, Serracino Inglott A, LaFerla G. Practical implications of pharmacogenetics in antidepressant treatment: the case of amitriptyline. Virtual poster symposium; 2017 May 17–18; American College of Clinical Pharmacy, Lenexa.

x. Mifsud Buhagiar L, Grech A, Serracino Inglott A, LaFerla G. Pharmacogenetic implications in antidepressant treatment. Presentation delivered at the University of Malta KSU Research Days; 2017 Mar 13–15; Msida, Malta.

xi. Mifsud Buhagiar L, Grech A, Serracino Inglott A, LaFerla G. Practical implications of pharmacogenetics in antidepressant treatment. Poster presented at the Festival of Genomics; 2017 Jan 31 – Feb 1; Excel, London.

xii. Mifsud Buhagiar L. Clinical implications of pharmacogenetics in psychiatric treatment – The case of amitriptyline. Oral presentation delivered to the Pharmacogenomics Working Party of the European Medicines Agency; 2016 Dec 12; Virtual meeting.

Appendices

A. Permission from the Journal of the Brazilian Chemistry Society for Figure 1-1

PubliSBQ
Sociedade Brasileira de Química

Impact Factor (JCR) 2017 = 1.444
Phone:+55 -19-3521-3151
office@jbcs.sbq.org.br
Link at http://jbcs.sbq.org.br
Online submission
Share our Articles

Journal of the Brazilian Chemical Society

Form for requesting *Copyright & Permissions*

Luana Mifsud Buhagiar
Department of Pharmacy, University of Malta

Dear Dr. Maria Suzana P. Francisco

I am requesting permission to reproduce a portion of the following JBCS research article:

Rafael Linden; Marina Venzon Antunes; Ana Luiza Ziulkoski; Maína Wingert; Paula Tonello; Mladen Tzvetkov; André Arigony Souto Rafael Linden; Marina Venzon Antunes; Ana Luiza Ziulkoski; Maína Wingert; Paula Tonello; Mladen Tzvetkov; André Arigony Souto; ***Determination of Amitriptyline and its Main Metabolites in Human Plasma Samples using HPLC-DAD: Application to the Determination of Metabolic Ratios after Single Oral Dose of Amitriptyline***; Braz. Chem. Soc. **2008**, *1*, 35.

http://dx.doi.org/10.1590/S0103-50532008000100007
Figure 1: Main metabolic pathways of amitriptyline. Major routes are shown in bold.

This request is for permission to include the above content as part of the following project that I am preparing:

In particular, I am requesting permission to include Figure 1 in the introductory chapter of my PhD thesis about clinical implications of pharmacogenetics in psychiatry.

It is for a non-exclusive, irrevocable, and royalty-free permission, and it is not intended to interfere with other uses of the same work by SBQ.

I would be pleased to include in the references a full citation above, and other acknowledgement as you might request, as an opportunity for JBCS will reach new readers and potential authors.

I would greatly appreciate your permission. If you require any additional information, do not hesitate to contact me at the address and number above.

Sincerely,

Luana Mifsud Buhagiar

==

***JBCS Permission is hereby granted*:** **Date: August 10, 2018**

Dr. Maria Suzana P. Francisco
PubliSBQ Editorial Manager

B. Genomic DNA extraction and CYP450 genotyping experimentation

Genomic DNA extraction and CYP450 genotyping

Experimentation at the BioDNA Lab, Malta

AccuPrep® Genomic DNA extraction kit was utilised for extracting DNA from whole blood samples through the following steps:

- 1.5 mL clean tubes, with 20 µL of Proteinase K, a broad-spectrum serine protease for digesting proteins in the samples;
- Addition of 200 µL of whole blood and 200 µL of binding buffer;
- Spinning in Vortex-Genie 2 (Scientific Industries, US), then incubated at 60˚C for 10 min in Jencons-PLS digital heating block (Jencons-PLS, UK);
- Isopropanol, 100 µL, added, pipetting for proper mixing;
- Lysate transferred into the upper reservoir of a binding column tube and centrifuged in Eppendorf® Microcentrifuge 5415D (Eppendorf, Germany) at 8000 rpm for 1 minute, discarding the filtrate;
- Addition of 500 µL of Washing Buffer 1 and recentrifuged at 8000 rpm for 1 minute, disposing the filtrate;
- Addition of 500 µL of Washing Buffer 2, centrifuged, filtrate discarded, and recentrifuged at 12000 rpm for 1 minute;
- Binding column transferred to a clean 1.5 mL tube;
- Addition of 40 µL Elution Buffer, left standing for 5 minutes, and centrifuged at 8000 rpm for 1 minute, discarding column;
- Eluted genomic DNA labelled and stored at 4°C for later analysis.

BuccalQuick™ extraction kit was utilised for extracting DNA from buccal swabs, through the following steps:

- Extraction Buffer prepared by transferring 300 µL BQ-Solution and 7 µL Enzyme Mix (volumes calculated per number of samples) into a tube, mixed by vortexing;
- 300 µL of Extraction Buffer transferred to each 2 mL tube labelled with sample ID;
- Swab head placed into the Extraction Buffer in the tube and twisted vigorously 8-10 times, pressing the swab head against the tube wall before discarding;
- Tube capped and vortexed for 10 seconds at high speed, then incubated at 55˚C for 5 min;
- Tube heated in heat block at 95˚C for 3 min, and final sample stored at 4˚C.

The extracted genomic DNA was intended for further processing in genotyping *CYP2D6* and *CYP2C19*, starting with the Polymerase Chain Reaction (PCR) steps described hereafter.

I. PCR Amplification

PCR Reaction Mix A and PCR Reaction Mix B were prepared as per the below calculation.

Master Mix = 18 µL x (number of samples/controls) x 1.1* = µL
PCR-P A or B = 2 µL x (number of samples/controls) x 1.1* = µL
*Adjustment for pipetting error

The volumes of Master Mix and PCR-P (containing PCR primers for the respective alleles tested) were mixed. Two labelled PCR tubes were allocated for each sample to be tested. One tube had 20µL of PCR Reaction Mix A added while the other had 20µL of PCR Reaction Mix B added, together with 2 µL of DNA. For the negative control, DNA was replaced by 2 µL of nuclease-free water. Each tube was placed in the Eppendorf® PCR thermal cycler with the following program: 1 cycle at 94 ˚C for 2 minutes; 35 cycles at 94 ˚C for 20 seconds then 53 ˚C for 30 seconds then 72 ˚C for 30 seconds; 1 cycle 72 ˚C for 1 minute; hold at 4 ˚C.

II. PCR Product Clean-Up

The PCR Clean-Up (C-UP) Mix was prepared as per the below calculation.

C-UP Buffer = 9 µL x (number of PCR tubes) x 1.2* = µL
C-UP1 = 1 µL x (number of PCR tubes) = µL
C-UP2 = 1 µL x (number of PCR tubes) = µL
*Adjustment for pipetting error

To each of a set of newly labelled tubes, 11 µL C-UP Mix were added. The PCR product was spinned and 5 µL added to each tube containing the C-UP Mix, followed by gentle mixing and spinning. The tubes were then incubated in the thermal cycler with the following programme: 1 cycle at 37 ˚C for 25 minutes; 1 cycle at 95 ˚C for 5 minutes; hold at 4 ˚C.

III. Genotyping Reaction

Shifted Termination Assay (STA) Mix A and Mix B were prepared as per the below calculation.

ST-F Mix: 11 µL x (number of C-UP tubes +1*) x 1.1** = µL
DP-A or B: 2 µL x (number of C-UP tubes +1*) x 1.1** = µL
*For controls **Adjustment for pipetting error

The ST-F Mix (reagent mix for allele detection) and DP-A or B (detection primers for the respective alleles tested) volumes were mixed to produce STA Mix A and STA Mix B. Two labelled PCR tubes were allocated for each sample to be tested, and two control tubes included. One tube had 13µL of STA Mix A added while the other had 13µL of STA Mix B added, together with 5 µL of the corresponding C-UP treated PCR-product. CTL-A and CTL-B solutions, 5 µL, were used for the controls, consisting of control DNA for the respective alleles under study. Following gentle mixing and spinning, the tubes were placed in the thermal cycler with the following programme: 1 cycle at 94 ˚C for 1 minute; 20 cycles at 94 ˚C for 20 seconds then 50 ˚C for 45 seconds then 72 ˚C for 10 seconds; hold at 4˚C. Through

the extension of primers by multiple bases, STA technology enhances signal strength and fragments size, allowing distinction of variants from wild-type during fragment analysis.

IV. Sample Loading

Loading buffer, 15 µL, was added to the wells of the sequencer adapter plate of the 3130 Genetic Analyser, followed by 5 µL of the STA reaction products being transferred into the wells. The plate was loaded into the sequencer and the pre-setup data collection programme run.

Figure C-1 shows preliminary results obtained during the first trials for *CYP2D6* Tube B, targeting alleles *6, *9, *10, and *17. Working with blood samples facilitated interpretation of the genotyping results. Figure C-2 shows the *CYP2C19* results obtained for a blood sample, whereby the lower signals calling for *5 (Tube A) and *7 and *17 (Tube B), hinder direct assignment of the diplotype, necessitating further analysis of the differences in peak heights between the sample and negative control. Reproducing the outcomes as images in this text is challenging since differentiation is facilitated by zooming on the y-axis. The graphs are presented in the following order: Blank, Negative Control, Positive Control, and finally the result for the extracted DNA. Results for *CYP2D6* genotyping of the blood sample illustrated in Figure C-3, show similarly low signals for *41 and also for *4 (Tube A). The latter could be interpreted as heterozygous with both A and G (i.e. *CYP2D6**1/*4), however due to the presence of similar peaks obtained in the negative control, such interpretation cannot be made with confidence.

Figure C-1: Preliminary experimentation outcome for *CYP2D6* Tube B

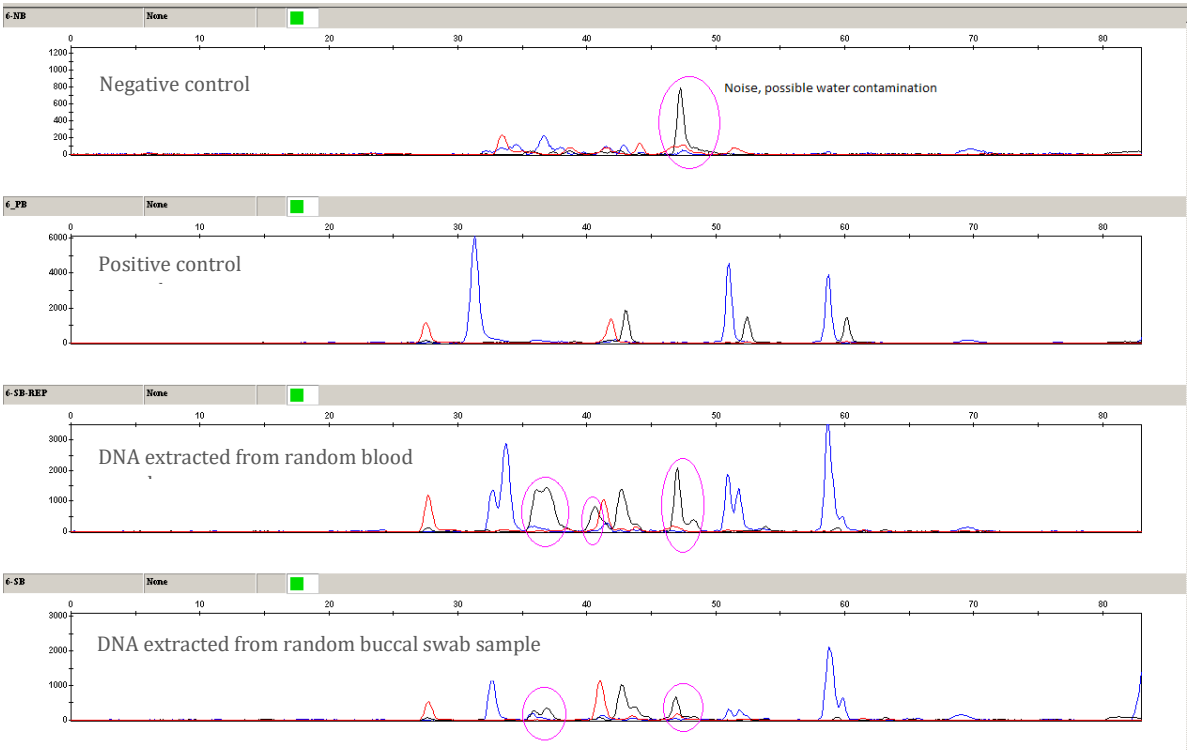


Figure C-2: Result for *CYP2C19* genotyping of DNA extracted from individual blood sample

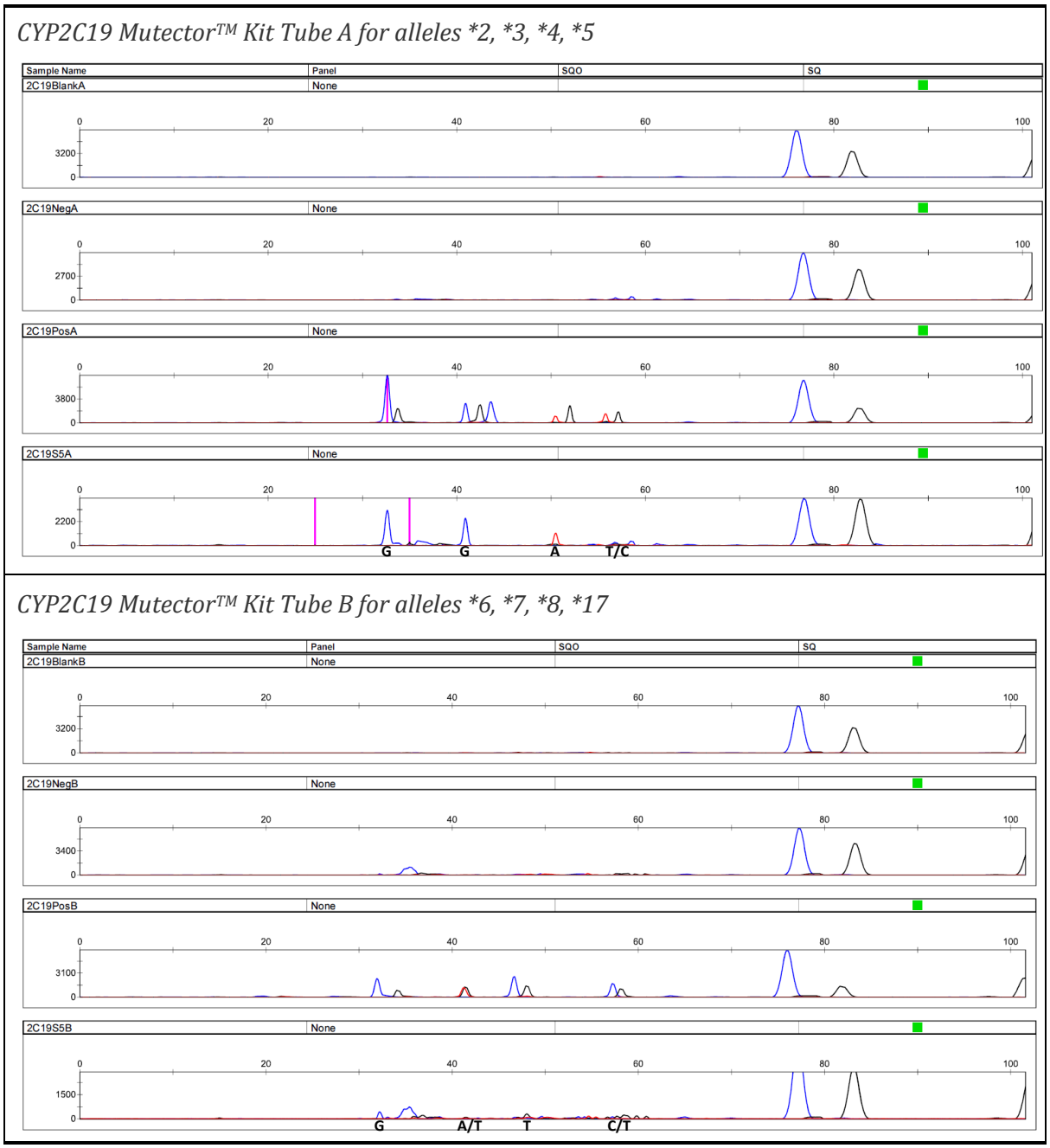
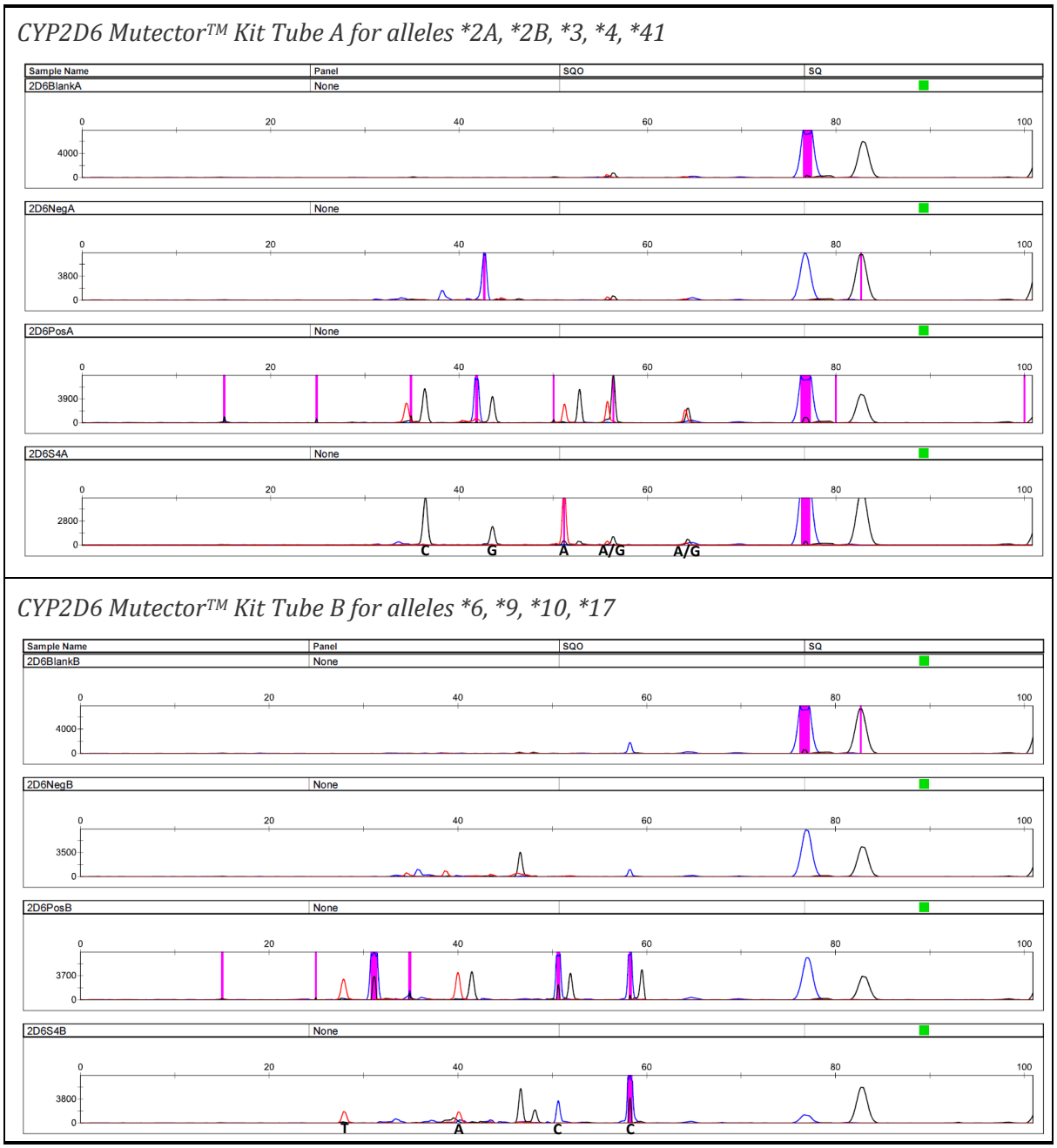


Figure C-3: Result for *CYP2D6* genotyping of DNA extracted from individual blood sample



C. University Research Ethics Committee (UREC) Approval

L-UNIVERSITÀ TA' MALTA

Msida – Malta
Skola Medika
Sptar Mater Dei

UNIVERSITY OF MALTA

Msida – Malta
Medical School
Mater Dei Hospital

Ref No: **23/2017**

Friday 28th July 2017

Ms. Luana Mifsud Buhagiar
Hibi
Hal-Bordi Street
Lija, LJA1623

Dear Ms. Luana Mifsud Buhagiar,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

Clinical Implications of Pharmacogenetics in Psychiatric Treatment

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Dr. Mario Vassallo
Chairman
Research Ethics Committee

D. Patient Information Sheets

INFORMATION SHEET

I, Luana Mifsud Buhagiar, a pharmacist, am currently undertaking a PhD at the Department of Pharmacy, University of Malta, with a research project entitled '*Clinical implications of pharmacogenetics in psychiatric treatment*' under the supervision of Prof. Godfrey Laferla, Dean of the Faculty of Medicine and Surgery, University of Malta, co-supervised by Prof. Anthony Serracino Inglott, Professor at the Department of Pharmacy, University of Malta, and advised by Dr. Anton Grech, Chairman of Psychiatry, Department of Health, Malta.

You have been identified to participate in this research; the protocol is described below.

Aim of the study and potential benefits

Several patients may not benefit from psychiatric treatment or incur severe side-effects. The effectiveness and tolerability of anti-depressants may be affected by metabolic enzyme activity which influences the drug blood concentration. This research will determine the functioning of these enzymes so that your clinician, backed by genotype-phenotype data and pharmacogenetic recommendations to complement the clinical picture, shall be in a better position to individualise therapy to pursue a more satisfactory outcome.

Your involvement

: Be examined by an independent specialist appointed by the Commissioner for the Promotion of Rights of Persons with Mental Disorders to certify your capability to give free and informed consent and that the expected benefits of the research are likely to outweigh any potential harm to yourself, according to the Mental Health Act (Chapter 525);
: Have a data collection sheet completed by myself via access to your medical records and through your contribution in rating scales;
: Have blood sample and buccal swab taken *once* by a medical professional, which may be subject to experimental testing including genotyping and drug blood concentration determination;
: Be followed up after the intervention, with potential treatment plan revision, as deemed necessary in terms of the results obtained.

Important information

Participation in this research is entirely voluntary and refusal to participate will not affect the treatment you receive. You may discontinue participation at any time without prejudice. Information gathered will be kept strictly confidential according to the Data Protection Act (Chapter 440).

If you agree to participate in this research, contact will be coordinated with the undersigned to schedule an appointment with the independent specialist and signing of the consent form once declared eligible.

Thank you in advance for your cooperation.

Luana Mifsud Buhagiar
B.Pharm. (Hons) M.Pharm. (Melit.)
luana.mifsud-buhagiar.06@um.edu.mt / 79709140

INFORMAZZJONI GĦALL-PAZJENT

Jiena, Luana Mifsud Buhagiar, spiżjara, bħalissa qed insewgi Dottorat fid-Dipartiment tal-Farmaċija ta' l-Università ta' Malta billi naħdem fuq proġett ta' riċerka intitolat '*Clinical implications of pharmacogenetics in psychiatric treatment*' taħt is-superviżjoni priniċipali tal-Professur Godfrey Laferla, Dekan tal-Fakultà tal-Mediċina u l-Kirurġija fl-Università ta' Malta u s-superviżjoni tal-Professur Anthony Serracino Inglott, Professur fid-Dipartiment tal-Farmaċija fl-Università ta' Malta, f'konsultazzjoni ma' Dr. Anton Grech, Chairman tad-Dipartiment tal-Psikjatrija fid-Dipartiment tas-Saħħa ta' Malta.

Inti ġejt identifikat/a biex tipparteċipa f'din ir-riċerka; il-protokoll huwa deskritt hawn taħt.

L-għan tar-riċerka u l-benefiċċji potenzjali

Diversi pazjenti ma jiksbux benefiċċju mit-trattamenti psikjatriċi jew jintlaqtu minn effetti sekondarji severi. L-effettività u t-tollerabilità ta' l-antidipressanti jistgħu jiġu affettwati mill-attività ta' enżimi metaboliċi li jinfluwenzaw l-konċentrazzjoni tal-mediċina fid-demm. Din ir-riċerka se tiddetermina l-funzjonament ta' dawn l-enżimi sabiex il-konsulent tiegħek, appoġġjat minn tagħrif rigward il-ġenotip-fenotip tiegħek u rakkomandazzjonijiet farmakoġenetiċi li jikkumplimentaw il-preżentazzjoni klinika, ikun f'pożizzjoni aħjar biex jindividwalizza t-terapija għal riżultatati aktar sodisfaċenti.

L-involviment tiegħek

: Tiġi eżaminat minn speċjalista indipendenti maħtur mill-Kummissarju għall-Promozzjoni tad-Drittijiet ta' Persuni b'Diżordni Mentali biex jiċċertifika li tista' tagħti kunsens ħieles u konsapevoli u li l-benefiċċji mistennija mir-riċerka x'aktarx li jkunu bilwisq akbar minn kull ħsara potenzjali li tista' ssirlek, skond l-Att dwar is-Saħħa Mentali (Kapitolu 525);
: Timtela dokumentazzjoni minni, permezz ta' aċċess għar-rekords mediċi tiegħek u permezz tal-kontribut tiegħek fi skali ta' klassifikazzjoni;
: Jittiħdulek kampjun tad-demm u ċelluli tal-ħalq *darba* minn professjonist mediku, li jistgħu jkunu soġġetti għal-ittestjar sperimentali li jinkludi ġenotipaġġ u d-determinazzjoni tal-konċentrazzjoni tal-mediċini fid-demm;
: Tiġi segwit, bil-possibilita' ta' reviżjoni fil-pjan tal-kura, kif jitqies meħtieġ fir-rigward tar-riżultati miksuba.

Informazzjoni importanti

Il-parteċipazzjoni tiegħek f'din ir-riċerka hija kompletament volontarja u t-trattament tiegħek ma jiġi affettwat bl-ebda mod jekk inti tirrifjuta. Inti tista' tieqaf milli tipparteċipa, fi kwalunkwe ħin, mingħajr ebda preġudizzju. L-informazzjoni miġbura tibqa' strettament kunfidenzjali skond l-Att dwar il-Protezzjoni u l-Privatezza tad-Data (Kap. 440).

Jekk taċċetta li tipparteċipa f'din ir-riċerka, ser ikun koordinat kuntatt mal-hawn taħt iffirmata għall-iskedar t'appuntament ma' l-ispeċjalista indipendenti u l-iffirmar tal-formola tal-kunsens ladarba ddikjarat/a eliġibbli.

Grazzi bil-quddiem tal-kooperazzjoni tiegħek.

Luana Mifsud Buhagiar
B.Pharm. (Hons) M.Pharm. (Melit.)
luana.mifsud-buhagiar.06@um.edu.mt / 79709140

INFORMATION SHEET

I, Luana Mifsud Buhagiar, a pharmacist, am currently undertaking a PhD at the Department of Pharmacy, University of Malta, under the supervision of Prof. Godfrey Laferla, Dean of the Faculty of Medicine and Surgery, University of Malta and Prof. Anthony Serracino Inglott, Professor at the Department of Pharmacy, University of Malta.

You have been identified to participate in my research with the protocol described below.

Aim of the study and potential benefits

The effectiveness and tolerability of amitriptyline may be affected by metabolic enzyme activity which influences the drug blood concentration. This study will determine the functioning of these enzymes so that your clinician, backed by genotype-phenotype data and pharmacogenetic recommendations to complement the clinical picture, shall be in a better position to individualise therapy to pursue a more satisfactory outcome.

Your involvement

: Consider giving free and informed consent upon discussing the research with your respective clinician;
: Have a data collection sheet completed by myself via access to your medical records and through your contribution in rating scales;
: Have blood sample and buccal swab taken *once* by a medical professional, which may be subject to experimental testing including genotyping and drug blood concentration determination;
: Be followed up after the intervention, with potential treatment plan revision, as deemed necessary in terms of the results obtained.

Important information

Participation in this study is entirely voluntary and refusal to participate will not affect the treatment you receive. You may discontinue participation at any time without prejudice. Information gathered will be kept strictly confidential according to the Data Protection Act (Chapter 440).

Thank you in advance for your cooperation.

Luana Mifsud Buhagiar
B.Pharm. (Hons) M.Pharm. (Melit.)
luana.mifsud-buhagiar.06@um.edu.mt / 79709140

INFORMAZZJONI GĦALL-PAZJENT

Jiena, Luana Mifsud Buhagiar, spiżjara, bħalissa qed insewgi Dottorat fid-Dipartiment tal-Farmaċija ta' l-Università ta' Malta taħt is-superviżjoni tal-Professur Godfrey Laferla, Dekan tal-Fakultà tal-Mediċina u l-Kirurġija fl-Università ta' Malta u tal-Professur Anthony Serracino Inglott, Professur fid-Dipartiment tal-Farmaċija fl-Università ta' Malta.

Inti ġejt identifikat/a biex tipparteċipa fir-riċerka tiegħi bil-protokoll deskritt hawn taħt.

L-għan tar-riċerka u l-benefiċċji potenzjali

L-effettività u t-tollerabilità ta' *amitriptyline* jistgħu jiġu affettwati mill-attività ta' enżimi metaboliċi li jinfluwenzaw il-konċentrazzjoni tal-mediċina fid-demm. Din ir-riċerka se tiddetermina l-funzjonament ta' dawn l-enżimi sabiex il-konsulent tiegħek, appoġġjat minn tagħrif rigward il-ġenotip-fenotip tiegħek u rakkomandazzjonijiet farmakoġenetiċi li jikkumplimentaw il-preżentazzjoni klinika, ikun f'pożizzjoni aħjar biex jindividwalizza t-terapija għal riżultatati aktar sodisfaċenti.

L-involviment tiegħek

: Tikkunsidra li tagħti kunsens liberu u infurmat meta tiddiskuti r-riċerka mal-konsulent rispettiv tiegħek;
: Timtela dokumentazzjoni minni, permezz ta' aċċess għar-rekords mediċi tiegħek u permezz tal-kontribut tiegħek fi skali ta' klassifikazzjoni;
: Jittiħdulek kampjun tad-demm u ċelluli tal-ħalq *darba* minn professjonist mediku, li jistgħu jkunu soġġetti għal-ittestjar sperimentali li jinkludi ġenotipaġġ u d-determinazzjoni tal-konċentrazzjoni tal-mediċini fid-demm;
: Tiġi segwit, bil-possibilita' ta' reviżjoni fil-pjan tal-kura, kif jitqies meħtieġ fir-rigward tar-riżultati miksuba.

Informazzjoni importanti

Il-parteċipazzjoni tiegħek f'din ir-riċerka hija kompletament volontarja u t-trattament tiegħek ma jiġi affettwat bl-ebda mod jekk inti tirrifjuta. Inti tista' tieqaf milli tipparteċipa, fi kwalunkwe ħin, mingħajr ebda preġudizzju. L-informazzjoni miġbura tibqa' strettament kunfidenzjali skond l-Att dwar il-Protezzjoni u l-Privatezza tad-Data (Kap. 440).

Grazzi bil-quddiem tal-kooperazzjoni tiegħek.

Luana Mifsud Buhagiar
B.Pharm. (Hons) M.Pharm. (Melit.)
luana.mifsud-buhagiar.06@um.edu.mt / 79709140

E. Patient Consent Forms

CONSENT FORM

I am a Maltese citizen over eighteen (18) years of age.

I have been asked to participate in a research study entitled:

'Clinical implications of pharmacogenetics in psychiatric treatment'

The purpose and details of the study have been explained to me by *Luana Mifsud Buhagiar*, and any questions which I raised have been adequately clarified.

I give my consent to the research team to access my medical records, make the appropriate observations, take the necessary samples for experimental testing, including genotyping, discuss results with my clinicians for potential treatment plan revision, and be followed up.

Similar to other clinical tests, blood drawing presents a small risk of complications including pain, bruising, and swelling. I am aware of the inconveniences which this may cause.

I consent to the retention of my samples for as long as they are considered useful for research purposes. I understand that the data may be used for medical or scientific rationales and that the results achieved may be reported or published; however, I shall not be personally identified in any way, without my written permission. Under the Data Protection Act I have the right to access, rectify and erase data concerning myself.

I am under no obligation to participate in this study and am doing so voluntarily. I am not receiving any remuneration for participating in this study. I may withdraw from the study at any time, without giving any reason. This will not affect in any way the care and treatment normally given to me.

I understand that any complications and/or adverse effects which may arise during or as a consequence of the study will be recorded and any treatment which this may entail will be given within the Government Health Services.

In case of queries during the study I may contact:

Luana Mifsud Buhagiar I.D. 392288M
Chief Investigator
luana.mifsud-buhagiar.06@um.edu.mt / 79709140

Signature _____

Name of participant

_____

I.D. of participant _____

Signature of participant _____

Date _____

FORMULA TAL-KUNSENS

Jien/a ċittadin/a Malti/ja u għalaqt tmintax (18) –il sena.

Ġejt mitlub/a nieħu sehem fi studju bl-isem ta':

'Clinical implications of pharmacogenetics in psychiatric treatment'

L-għan u d-dettalji tar-riċerka spjegathomli *Luana Mifsud Buhagiar* li ċċaratli wkoll xi mistoqsijiet li għamilt.

Jiena nagħti l-kunsens tiegħi lit-tim tar-riċerka biex jaċċessa r-rekords kliniċi tiegħi, jagħmel l-osservazzjonijiet neċessarji, jieħu l-kampjuni meħtieġa għal ittestjar sperimentali, ġenotipaġġ inkluż, jiddiskuti r-riżultati mat-tobba tiegħi għal reviżjoni potenzjali fil-pjan tal-kura, u niġi segwit. Simili għal testijiet kliniċi oħra, it-teħid tad-demm għandu riskju żgħir ta' kumplikazzjonijiet inkluż uġigħ, tbenġil, u nefħa. Nifhem li dan jista' jkun ta' skomdu għalija.

Jiena nagħti l-kunsens biex il-kampjuni tiegħi jinżammu sakemm ikunu kkunsidrati utli għal skopijiet ta' riċerka. Nifhem li l-informazzjoni tista' tintuża għal raġunijiet mediċi jew xjentifiċi u r-riżultati miksuba jistaw jiġu ppubblikat jew irrappurtati bil-miktub; madankollu, b'edba mod ma nista' jiena nkun identifikat personalment, mingħajr kunsens tiegħi bil-miktub. Taħt l-Att dwar il-Protezzjoni u l-Privatezza tad-*Data* għandi d-dritt naċċessa, nirrettifika u nħassar id-*data* li tirrigwarda lili.

Jiena m'għandi l-ebda dmir li nieħu sehem f'din ir-riċerka u qed nagħmel hekk minn rajja. Jiena mhux qed nitħallas biex nieħu sehem f'dan l-istudju. Jiena nista', meta irrid, ma nkomplix nieħu sehem fl-istudju, u mingħajr ma' nagħti raġuni. Jekk nagħmel hekk, xorta nibqa' nieħu l-kura li ssoltu tingħatali.

Jiena nifhem li jekk ikun hemm xi kumplikazzjonijiet jew effetti kollaterali waqt jew b'konsegwenza ta' l-istudju, dawn jiġu mniżżla bil-miktub u jekk ikun hemm bżonn xi kura, tiġi mgħotija mis-Servizzi Nazzjonali tas-Saħħa.

F'kas ta' diffikulta' waqt l-istudju, nista' nikkuntatja lil:

Luana Mifsud Buhagiar I.D. 392288M
Persuna responsabbli għal din ir-riċerka
luana.mifsud-buhagiar.06@um.edu.mt / 79709140

Firma _____

Isem tal-parteċipant

_____

Numru ta' l-identità tal-parteċipant _____

Firma tal-parteċipant _____

Data _____

CONSENT FORM

I am a Maltese citizen over eighteen (18) years of age.

I have been asked to participate in a research study which aims to investigate the implications of pharmacogenetics in treatment with amitriptyline.

The purpose and details of the study have been explained to me by *Luana Mifsud Buhagiar*, and any questions which I raised have been adequately clarified.

I give my consent to the research team to access my medical records, make the appropriate observations, take the necessary samples for experimental testing, including genotyping, discuss results with my clinicians for potential treatment plan revision, and be followed up.

Similar to other clinical tests, blood drawing presents a small risk of complications including pain, bruising, and swelling. I am aware of the inconveniences which this may cause.

I consent to the retention of my samples for as long as they are considered useful for research purposes. I understand that the data may be used for medical or scientific rationales and that the results achieved may be reported or published; however, I shall not be personally identified in any way, without my written permission. Under the Data Protection Act I have the right to access, rectify and erase data concerning myself.

I am under no obligation to participate in this study and am doing so voluntarily. I am not receiving any remuneration for participating in this study. I may withdraw from the study at any time, without giving any reason. This will not affect in any way the care and treatment normally given to me.

I understand that any complications and/or adverse effects which may arise during or as a consequence of the study will be recorded and any treatment which this may entail will be given within the Government Health Services.

In case of queries during the study I may contact:

Luana Mifsud Buhagiar I.D. 392288M
Chief Investigator
luana.mifsud-buhagiar.06@um.edu.mt / 79709140

Signature _____

Name of participant

_____

I.D. of participant _____

Signature of participant _____

Date _____

FORMULA TAL-KUNSENS

Jien/a ċittadin/a Malti/ja u għalaqt tmintax (18) –il sena.

Ġejt mitlub/a nieħu sehem fi studju bil-għan li jinvestiga l-implikazzjonijiet farmakogenetiċi fit-trattament b'*amitriptyline*.

Id-dettalji tar-riċerka spjegathomli *Luana Mifsud Buhagiar* li ċċaratli wkoll xi mistoqsijiet li għamilt.

Jiena nagħti l-kunsens tiegħi lit-tim tar-riċerka biex jaċċessa r-rekords kliniċi tiegħi, jagħmel l-osservazzjonijiet neċessarji, jieħu l-kampjuni meħtieġa għal ittestjar sperimentali, ġenotipaġġ inkluż, jiddiskuti r-riżultati mat-tobba tiegħi għal reviżjoni potenzjali fil-pjan tal-kura, u niġi segwit. Simili għal testijiet kliniċi oħra, it-teħid tad-demm għandu riskju żgħir ta' kumplikazzjonijiet inkluż uġigħ, tbenġil, u nefħa. Nifhem li dan jista' jkun ta' skomdu għalija.

Jiena nagħti l-kunsens biex il-kampjuni tiegħi jinżammu sakemm ikunu kkunsidrati utli għal skopijiet ta' riċerka. Nifhem li l-informazzjoni tista' tintuża għal raġunijiet mediċi jew xjentifiċi u r-riżultati miksuba jistaw jiġu ppubblikat jew irrappurtati bil-miktub; madankollu, b'edba mod ma nista' jiena nkun identifikat personalment, mingħajr kunsens tiegħi bil-miktub. Taħt l-Att dwar il-Protezzjoni u l-Privatezza tad-*Data* għandi d-dritt naċċessa, nirrettifika u nħassar id-*data* li tirrigwarda lili.

Jiena m'għandi l-ebda dmir li nieħu sehem f'din ir-riċerka u qed nagħmel hekk minn rajja. Jiena mhux qed nitħallas biex nieħu sehem f'dan l-istudju. Jiena nista', meta irrid, ma nkomplix nieħu sehem fl-istudju, u mingħajr ma' nagħti raġuni. Jekk nagħmel hekk, xorta nibqa' nieħu l-kura li ssoltu tingħatali.

Jiena nifhem li jekk ikun hemm xi kumplikazzjonijiet jew effetti kollaterali waqt jew b'konsegwenza ta' l-istudju, dawn jiġu mniżżla bil-miktub u jekk ikun hemm bżonn xi kura, tiġi mgħotija mis-Servizzi Nazzjonali tas-Saħħa.

F'kas ta' diffikulta' waqt l-istudju, nista' nikkuntatja lil:

Luana Mifsud Buhagiar I.D. 392288M
Persuna responsabbli għal din ir-riċerka
luana.mifsud-buhagiar.06@um.edu.mt / 79709140

Firma _____

Isem tal-partiċipant

_____

Numru ta' l-identità tal-partiċipant _____

Firma tal-partiċipant _____

Data _____

F. Data Collection Forms

Patient Number ☐

Consultant _____

1 Patient Demographics and Social History

Age **Weight**	_____ _____	**Gender**	☐ Male ☐ Female
Marital status	☐ Single ☐ Married ☐ Widowed	☐ In a relationship ☐ Separated/Divorced	
Ethnicity	☐ Caucasian ☐ Other _____	**Smoking**	☐ Past ☐ Active ☐ Never
Nationality	☐ Maltese ☐ Other	**Gainful employment**	☐ Yes ☐ No
Illicit drug use	☐ Past ☐ Active ☐ Never	**Alcohol consumption**	☐ Regular ☐ Socially ☐ Never

2 Medical history and co-morbidities

Depression

Age at onset _____

Past hospitalisations related to depression or adverse events from its treatment	☐ Yes ☐ No
Family history of depression	☐ Yes ☐ No

OTHER DISORDERS *including* **Psychiatric / Endocrine / Cardiovascular / Gastrointestinal disorders**

---

3 Medications

Drug allergies

None ☐

Drug history *(antidepressants stopped)*

Generic name	*Dose*	*Stop date*	*Reason (if known)*

Current medications

Class	*Generic name*	*Dose*	*Dosage regimen*	*Start date (if known)*
Tricyclic antidepressant	Amitriptyline			

4 Samples

Buccal cells

Date of swab: _____

Bloods

Date and time withdrawn: _____

Date and time of last amitriptyline administration: _____

Patient Number ☐

1 Patient Demographics and Social History

Age **Weight**	_____ _____	**Gender**	☐ Male ☐ Female
Marital status	☐ Single ☐ Married ☐ Widowed	☐ In a relationship ☐ Separated/Divorced	
Ethnicity	☐ Caucasian ☐ Other _____	**Smoking**	☐ Past ☐ Active ☐ Never
Nationality	☐ Maltese ☐ Other	**Gainful employment**	☐ Yes ☐ No
Illicit drug use	☐ Past ☐ Active ☐ Never	**Alcohol consumption**	☐ Regular ☐ Socially ☐ Never

2 Medical history and co-morbidities

Neuropathic Pain

Date of onset

_____

Past hospitalisations related to pain or adverse events from its treatment	☐ Yes ☐ No
Family history of neuropathic pain	☐ Yes ☐ No

OTHER DISORDERS *including* **Psychiatric / Endocrine / Cardiovascular / Gastrointestinal disorders**

_____

3 Medications

Drug allergies

None ☐

_____

Drug history *(pain medication stopped)*

Generic name	*Dose*	*Stop date*	*Reason (if known)*

Current medications

Class	*Generic name*	*Dose*	*Dosage regimen*	*Start date (if known)*
Tricyclic antidepressant	Amitriptyline			

4 Samples

Buccal cells

Date of swab: _____

Bloods

Date and time withdrawn: _____

Date and time of last amitriptyline administration: _____