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**DISSERTATION**


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This dissertation was conducted at the Institute of Pharmacology with Toxicology and Clinical Pharmacology of the Faculty of Medicine/University of Prishtina, Prishtina/Kosovo and the Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University "Ss Kiril and Metodij", Skopje, Republic of Macedonia.

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**Acknowledgement**

I would like to express my utmost gratitude to Professor Nada Božina and Academician Aleksandar Dimovski for their continuous support, advice and encouragement with regard to this research. In addition, I am very grateful to the Ministry of Education, Science and Technology of Kosovo for supporting financially this scientific research.

I would like also to thank my family, especially my wife, my sons, Mom and Dad, for the continuous support they have given me throughout my time in PhD programme. I could not have done it without them.

**Abbreviations:**

| | |
|---|---|
| Adverse Drug Reactions | (ADR) |
| Angiotensin II receptor blockers | (ARBs) |
| Area Under the Curve | (AUC) |
| Aryl Hydrocarbon Receptor | (AHR) |
| Basic-Helix-Loop-Helix-PAS | (bHLH-PAS) |
| Clinical Pharmacogenetics Implementation Consortium | (CPIC) |
| Confidence Interval | (CI) |
| Constitutive Androstane Receptor | (CAR) |
| Cyclooxygenase - 2 | (COX-2) |
| Cytochrome P 450 | (CYP450 or CYPs) |
| Cytochrome P 450 2C19 | (CYP2C19) |
| Cytochrome P 450 2C9 | (CYP2C9) |
| Cytochrome P 450 3A5 | (CYP3A5) |
| Deoxyadenosine triphosphate | (dATP) |
| Deoxycytidine triphosphate | (dCTP) |
| Deoxyguanosine triphosphate | (dGTP) |
| Deoxyribonucleic acid | (DNA) |
| Deoxythymidine triphosphate | (dTTP) |
| Drug Absorption, Distribution, Metabolism and Excretion | (ADME) |
| Drug Metabolizing Enzymes | (DMEs) |
| Dutch Pharmacogenetics Working Group Guideline | (DWPG) |
| Epoxyeicosatrienoic Acids | (EETs) |
| European Medicines Agency | (EMA) |
| European Public Assessment Report | (EPAR) |
| Extensive Metabolizer | (EM) |
| Food and Drug Administration | (FDA) |
| Gas Chromatography-Mass Spectrometry | (GC-MS) |
| Health Canada or Santé Canada | (HCSC) |
| High Performance Liquid Chromatography | (HPLC) |
| Intermediate Metabolizer | (IM) |
| Kilodaltons | (kDA) |
| Liquid Chromatography-Mass Spectrometry | (LC-MS) |
| Nonsteroidal anti-inflammatory drug | (NSAIDs) |
| Once per day dosing of tacrolimus | (Tac-OD) |
| Peroxisome Proliferator-Activated Receptors | (PPARα) |
| Pharmaceuticals and the Medical Devices Agency of Japan | (PMDA) |
| Pharmacogenomics | (PGx) |
| Pharmacogenomics Knowledge Base association | (PharmGKB) |

| | |
|---|---|
| Polymerase Chain Reaction | (PCR) |
| Poor Metabolizer | (PMs) |
| Pregnane X Receptor | (PXR) |
| Protein C | (PROC) |
| Protein S | (PROS1) |
| Real-Time Polymerase Chain Reaction | (RT-PCR) |
| Restriction Fragment Length Polymorphism | (RFLP) |
| Ribonucleic acid | (RNA) |
| Selective serotonin reuptake inhibitor | (SSRI) |
| Single nucleotide polymorphisms | (SNPs) |
| Summary of Product Characteristics | (SmPCs) |
| Therapeutic drug monitoring | (TDM) |
| Tricyclic antidepressants | (TCAs) |
| Tris or Tris(hydroxymethyl)aminomethane + EDTA [500mM (pH 8.0) solution] + Acetic acid | (TEA buffer) |
| Twice-a-day dosing of tacrolimus | (Tac-BID) |
| UDP glucuronosyltransferase family 1 member A1 | (UGT1A1) |
| Ultra-Extensive Metabolizer | (UEM) |
| Vitamin K 2,3-epoxide reductase complex subunit 1 | (VKORC1) |

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# 1. Introduction

Evident variability among patients concerning pharmacokinetics and pharmacodynamics of the most often prescribed drugs in clinical practice is one of the major challenges nowadays (Ma and Lu 2011, Preissner, Hoffmann et al. 2013). Besides intrinsic (age, gender, weight, height, disease, organ impairment, ethnicity, race) and extrinsic factors that summarize information associated with the patient environment (use of other drugs, tobacco, alcohol, and food habits), this variability is also influenced by inherited polymorphism of genes that have an important impact on drug detoxifying enzymes, drug transporters and drug targets (Kantae, Krekels et al. 2017).

To date, a number of genetic variants of drug metabolizing enzymes, drug transporters, receptors, and different modulators (like nuclear receptors genes) have been discovered (Kim, Cheong et al. 2014). Hence, the presence of genetic aberrations regarding expression, regulation and activity of genes coding for Phase I and Phase II enzymes, membrane transport proteins and drug targets, may be a useful tool for understanding as well as predicting drug efficacy and/or toxicity related to drug use (Krasniqi, Dimovski et al. 2016; Maagdenberg, Vijverberg et al. 2016). This study, for the first time explored frequency and distribution of the most important variant alleles of drug metabolizing enzymes and drug target in Kosovo. This data is missing in the case of Kosovo's population. Kosovo is located in the heart of Balkan Peninsula. According to the 2011 census, Kosovo's population is 1,739,825, excluding the northern part of the country. 92.9% of Kosovo's inhabitants are ethnic Albanians. The rest includes minorities of Caucasian origin (Serbs 1.5%, Bosniaks 1.6%, and Gorani 0.6%) as well as of Asian origin (Turks 1.1% and Romani 0.5%) (Link 1) Characterization of the European Y-chromosome DNA haplogroups showed that in Kosovo's population there are present haplogroups that are characteristic for South-eastern and Western Europe. In addition, this data also proves different human migrations carrying mainly Neolithic and Bronze Age European ancestry (Link 2).

Polymorphic detoxifying enzymes of phase I metabolize nearly 59% of drugs cited in studies dealing with adverse drug reactions (ADR). Among these Phase I enzymes, CYPs constitute 75-86%. On the other hand, only 20% of drugs that are substrates of non-polymorphic enzymes have been part of ADR reports. These findings suggest that genetic variability in drug detoxifying enzymes is likely to be an important factor contributing to the incidence of ADRs. A number of published studies have

pointed out the importance of pharmacogenomics in decreasing and/or preventing ADRs (Phillips, K. A. et al 2001).

Besides, genomic data has become important in the assessment of the efficacy and safety of a medicinal product in the course of its regulatory approval.

Information on genomic markers is increasingly included in product information. By including pharmacogenomics information into the SPC (eng. Summary of Product Characteristics -SmPCs), regulatory authorities wish to point out the importance of the role of genomic variability in pharmacotherapy. Likewise, information regarding the category that the pharmacogenetics information belongs to, is also being included, i.e. whether testing is mandatory, recommended, or of an informational nature.

Drug's regulatory agencies, such as the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) in the US proposed these genetic variants (polymorphisms) as biomarkers for therapeutic guidance or safety warnings (Ehmann F et al 2015).

The link between pharmacogenomics and pharmacovigilance also plays an important role in monitoring the incidence of side effects in the post-marketing period.

At this point, the European Medicine Agency policy is oriented towards the implementation of pharmacogenetics findings in drug development, especially in pharmacokinetic characterization of drugs as a key step in designing and performing drug development, as well as in conducting drug evaluation studies (Ehmann, Caneva et al. 2014). Several medical products that have been authorized by the EMA in their product information SmPC contain pharmacogenetics (PGx) information (Link 3). Out of the total number, 15% of such products contain pharmacogenetics data in SmPC that have a direct impact in patient's treatment, showing that pharmacogenetics nowadays has become an essential part in the development and post-marketing phase for numerous of drugs. PGx also has a significant role in the management of drug's benefits and risks in clinical practice (Link 4).

In the US, out of approved drug labels from Food and Drug Administration (FDA) mentioning human genomic biomarkers, 62,0% contain information about the most common CYPs polymorphisms such as CYP2C9 (7.0%; warfarin, NSAIDs, phenytoin), CYP2C19 (17.0%; clopidogrel, PPIs, barbiturates, voriconazole), CYP2D6 (35.0%; tamoxifen, clozapine, risperidone, metoprolol, codeine etc.), as well as about gene polymorphisms of Phase II enzymes

such as UGT1A1 (nilotinib, irinotecan) and drug target VKORC1 (warfarin) (Foster and Sharp 2002). A total of 251 drugs has drug labels containing pharmacogenetic information.

Investigation regarding the rational use of drugs as well as optimization and personalization of pharmacotherapy has enabled the systematic identification of human genes performed within the Human Genome Project (Weinshilboum 2003, Green 2016).

In addition to revealing inter-individual differences in drug response, numerous investigations conducted on different ethnic groups also identified an existing association between genetic variants and disease susceptibility, drug metabolism and environmental response, as well as reported prevalence of these genetic variants in respective populations. As a result, human genome sequencing reinforced the interest of science in biological differences between races and ethnic groups (Mersha and Abebe 2015; Kaplun, Hogan et al. 2016).

Data generated from genome resequencing studies has shown that the majority of genetic variability in human population is attributed more to intra-population differences rather than interethnic differences (Garcia-Martin 2008, Stranger, Stahl et al. 2011).

A recently published study examined worldwide patterns of genetic diversity and signals of natural selection for ADME (drug absorption, distribution, metabolism and excretion) human genes. This study comprised 283 drug metabolizing enzymes (DMEs) in 62 different ethnic groups. The study's findings suggest that genetic variants concerning pharmacokinetic parameters such as drug absorption, distribution, metabolism and excretion could have an impact on the intra-population heterogeneity in drug response (Li, Zhang et al. 2011).

In clinical terms, genetic variants of Phase I enzymes CYP2C9, CYP2C19, and CYP3A5, as well as drug target VKORC1, are among the most important. Their function - catalytic activity and prevalence of variant alleles in European populations have been studied intensively. Given the paucity of data on genetic variants of these enzymes and drug target in Kosovo, in this study, we aimed to assess for the first time frequency distribution of these gene variants in Kosovo's population.

## 1.1. Cytochrome P450 (CYP450) enzyme system

The human cytochrome P450 (CYP) superfamily comprises a large and diverse group of enzymes (Guengerich FP, 2008). In humans, out of 115 CYP genes, 57 genes are active and the rest are pseudogenes (Sim, S. C. et al 2010). Most of the CYPs are found in the liver but certain amounts are also found in other parts of the body, such as intestines, lung, and kidney (Bozina N et al. 2009). Liver CYPs are composed of three main families of enzymes named CYP1, CYP2 and CYP3. In addition, within CYPs there is also the CYP4 family, whose major activity is its contribution in detoxification of eicosanoids, fatty acids and of a small number of xenobiotics (Simpson, 1997; Honkakoski, P. and M. Negishi 2000). CYP enzymes catalyze biotransformation of drugs, environmental xenobiotics, toxins, prostaglandins, fatty acids, steroidal hormones and lipids within Phase I of metabolism (Gueguen, Mouzat et al. 2006; Pandey and Fluck 2013). Within 57 active genes in humans that encode important detoxifying enzymes, the CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 metabolize about 90 %of drugs including clopidogrel, warfarin, NSAIDs, phenytoin, tacrolimus, tamoxifen, codeine, PPIs, barbiturates, and others (Lynch T et al. 2007; Zhou et al, 2009). CYPs also play an important role in the metabolism of the arachidonic acid. CYP1A2, CYP2C, and CYP2J2 metabolize the arachidonic acid into epoxyeicosatrienoic acids (EETs) which possess anti-inflammatory, vasodilating, anti-thrombotic, anti-apoptotic, natriuretic, as well as cardioprotective effects (Zordoky and El-Kadi 2010). The two main characteristics of CYPs' function include drug metabolism and bioactivation. They are involved mainly in the oxidation reactions serving as a monooxygenases, peroxydases and oxydases but can also serve as catalysts in reduction reactions. They account for approximately 75% of the total number of different metabolic reactions (Johansson I, 2011). A number of xenobiotics undergo detoxification processes or activation into reactive intermediate substances (Thier R et al, 2003). CYPs play a significant role in the duration and intensity of drug's action, detoxification of xenobiotics as well as in their transformation into toxic substances, mutagens and carcinogens (Nebert DW, 2001; Wogan et al. 2004). The inter-individual difference concerning the amount and activity of CYP450 enzymes, is due to the genetic and environmental factors. The genetic factors that contribute to inter-individual variability of CYPs' activity are variant alleles with changed/impaired function comparing to "wild type" gene. Substitution in the aminoacid sequence of protein may increase or rather

decrease enzyme activity. Other factors that may have an influence in gene transcription and, subsequently, in the quantity of CYP detoxifying enzymes are drugs (rifampin, barbiturate), fruits (grapefruit, orange, cranberry, apple, mango), vegetables (cruciferous vegetables such as cabbage, watercress and broccoli as well as spinach, red pepper, tomato and carrot), herbal medicines (chamomile, gingko), habits (alcohol, smoking) and diseases (diabetes, hypothyroidism and hyperthyroidism) (Fujita K, 2004; Nahrstedt A et al, 2010; Hermann R et al 2012).

Increased enzyme activity may be consequence of gene duplications, which generates an overexpression of CYP enzymes (Haouala et al., 2011; Sadee W et al 2011). On the other hand, a reduced activity of CYPs may be due to mutations in genes coding for these enzymes, which blocks enzyme's synthesis, or produce inactive enzymes (Ingelman-Sundberg, M. 2004). In addition, CYPs decreased activity may result from the exposure to environmental factors such as xenobiotics or infectious diseases, which suppress CYP450 expression, or as a consequence of suppression or inactivation of the existing enzymes by these factors. In addition to various disease conditions (chronic hepatitis C, steatosis, alcoholic liver diseases and others), liver's CYPs' expression is also under the control of nuclear receptors. The CYP1 gene family is regulated by AhR (aryl hydrocarbon receptor), the CYP2 family is regulated by CAR (constitutive androstane receptor), the CYP3 family is regulated by PXR (pregnane X receptor), and PPARα (peroxisome proliferator-activated receptors) regulates the CYP4 family (Aleksunes and Klaassen 2012). Many drugs affect CYPs' activity causing clinically significant changes in the concentrations of co-administered drugs. This impact of some drugs on the activity of CYPs, known as pharmacokinetic drug–drug interactions, produces adverse effects that in some cases can be life threatening. Some of these drugs that influence CYP enzymes induce the ability of the specific pathway to metabolize drugs, whereas others inhibit it. With respect to CYPs induction, it may occur through five mechanisms (Fuhr, U. 2000). The CYP2E1 enzyme is selectively induced by ethanol primarily by mediating enzyme stabilization. The other mechanisms are likely mediated by intracellular receptors such as the peroxisome proliferator activated receptor (PPAR), aryl hydrocarbon (Ah) receptor, pregnane X receptor (PXR, rifampicin induction) and the constitutive androstane receptor (CAR, phenobarbital induction) (Fuhr 2000, Prakash, Zuniga et al. 2015). The Ah receptor belongs to the basic-helix-loop-helix-PAS (bHLH-PAS) family and is a transcription factor while the other receptors such as CAR, PXR, and PPAR belong to the ligand-

activated orphan nuclear receptors (Waxman 1999, Gonzalez 2008). Induction by drugs produces stimulation of the other drug metabolism and consequently increases or reduces its therapeutic effect (Zhou SF et al 2009).

CYPs inhibition can be reversible or irreversible. Irreversible inhibition is likely the more common mechanism. On a kinetic basis, reversible inhibition can be divided into competitive, noncompetitive, and uncompetitive inhibition. Competitive inhibition is a type of inhibition where both the inhibitor and the substrate (drug) compete for the same binding site within a specific CYP enzyme. On the other side, in noncompetitive inhibition, the inhibitor and the substrate bind in different sites of the same enzyme whereas in uncompetitive inhibition, inhibitory substance binds exclusively to a CYP enzyme that forms a single complex with the drug-substrate (Ito, Iwatsubo et al. 1998). According to some studies, CYPs' reversible inhibitors comprise drugs such as miconazole (CYP2C9), fluoxetine (CYP2D6), ciprofloxacin (CYP1A2) and itraconazole (CYP3A4) (O'Reilly, Goulart et al. 1992, Sager, Lutz et al. 2014, Dirix, Swaisland et al. 2016, Meyer, Proctor et al. 2016). Inhibition of CYPs activity by a specific drug will generate deterioration of the other drug metabolism, producing an increased or toxic effect of that drug. Therefore, CYPs' inhibition results in a genetically decreased expression of these enzymes (Sideras, 2010).

In the case of CYPs' induction or inhibition, the main inhibitors of CYP2C9 are fluconazole, amiodarone, isoniazid, valproic acid, fenofibrate, and sertraline, while the main inducers are barbiturates, carbamazepine, and rifampin. As far as CYP2C19 is concerned, its most important inhibitors are omeprazole, chloramphenicol, cimetidine, fluoxetine, and oxcarbamazepine, while carbamazepine, rifampicin, pentobarbital, prednisone, and norethindrone are its main inducers (Zhou SF et al. 2010). Finally, CYP3A5's major inhibitors are chlorpheniramine, cocaine, diltiazem, indinavir, lovastatin, while the most important inducers are drugs such as carbamazepine, efavirenz and glucocorticoids (Liu, Y. T., 2007).

## 1.2. Genetic variants of drug target *VKORC1*

VKORC1 (Vitamin K 2,3-epoxide reductase complex subunit 1) protein is the drug target of coumarin anticoagulants like warfarin and is encoded by *VKORC1* gene. Its function to convert

vitamin K epoxide into vitamin K is an essential step in the Vitamin K cycle. Genetic variants of *VKORC1* are responsible for partial or total warfarin resistance (Watzka, M. et al 2011). Thus, information regarding the presence of *VKORC1* genetic variant enables to determine the starting dose of warfarin and avoid of drugs' side effects (Buzoianu, Trifa et al. 2012).

## 1.3. CYP450 phenotypes

The presence of different variant alleles and metabolism intensity determine most often three categories of phenotypes and in some cases, such as CYP2C19 and CYP2D6 four phenotypes. First, a poor metabolizer (PMs) lacks both active alleles and is characterized by a decreased (or absence of) enzyme activity and, consequently, accumulation of different drug-substrates. This phenotype is an example of the autosomal recessive inheritance. The extensive metabolizer (EM) possesses two functional alleles and manifest expressed metabolism. This phenotype (EM) is present in the majority of population. In addition to EMs, the ultra-extensive metabolizer (UEM) is marked by amplification of the respective gene (carrying more than two copies of the active gene) and is associated with the rapid/ultrarapid detoxification of substrates. This feature is inherited in an autosomal dominant way. The last phenotype is the intermediate metabolizer (IM) which is the intermediate form of metabolizer and individuals belonging to this group carry one normal (wt-wild type) and one defective allele (Ingelman-Sundberg M et al 2007; Birdwell KA et al 2015). The determination of drug metabolism phenotype may be conducted by either phenotypisation or genotypisation.

### 1.3.1. Phenotypisation

Phenotypisation is accomplished by administration of a test drug whose metabolism is known to be strongly linked to the activity of a certain drug metabolizing enzyme and followed by calculation of the metabolic ratio (MR), which represents the ratio between the pattern drug (unchanged drug) and its metabolite measurement in serum or urine (Zanger UM et al 2004). The phenotyping process can be performed using high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS) (Tanaka E et al 2003; Trojan A et al 2012). An advantage of phenotyping in

comparison with genotyping is that phenotyping reveals drug-drug interactions or any other defect concerning drug metabolism in general. On the other hand, phenotyping's disadvantages include the complex protocol of drug testing, unreliability of results because of the disease impact, with particular emphasis on liver and kidney disease, as well as possible interaction with other substrates (Rost KL et al 1995; Swanson JR et al 1997). Table 1 lists some of the test drugs used in determining metabolic phenotype of the most important CYP450 detoxifying enzymes.

**Table 1. Test drugs for CYP450 phenotyping**

| Enzyme | Test drug for phenotyping |
|---|---|
| CYP 2C8 | bisphosphonates, ibuprofen, mycophenolic acid, pioglitazone |
| CYP 2C9 | warfarin, ibuprofen, phenytoin, cyclophosphamide |
| CYP 2C19 | clopidogrel, omeprazole, amitriptyline, mephenytoin, citalopram |
| CYP 2D6 | risperidone, tamoxifen, dextromethorphan, metoprolol, debrisoquine |
| CYP 3A4 | cyclosporine, doxorubicin, clarithromycin, midazolam |
| CYP 3A5 | chlorpheniramine, cocaine, diltiazem, indinavir, lovastatin, methadone, |
| CYP 1A2 | caffeine, clopidogrel, theophylline, warfarin |

## 1.4. Genotyping

Genotyping comprises the procedure of identification of CYP450 gene variants using diagnostic molecular methods such as polymerase chain reaction (PCR) or real-time polymerase chain reaction (RT-PCR). This procedure makes possible the identification of subjects that are carriers of two (homozygous for variant allele) or one (heterozygous for variant allele) variant allele and is manifested in a specific metabolic phenotype (Zhou SF, 2009a). These specific gene variants result in overexpression (subjects with more than two functional alleles), absence of certain active enzyme (subject that lack both active – wt- alleles) or generation of a defective protein that is characterized by a low catalytic activity (inactivating allele) (Garte S et Crosti F, 1999).

Different laboratory techniques are on disposal to perform screening of these genetic variants linked to the altered drug metabolism. At the beginning of genotyping, methods were based on multiplication of a specific segment of the gene of interest by PCR and thereafter on digestion of the multiplied DNA product using restriction endonucleases. Results were obtained by comparing the digestion product size from the amplified DNA segment to a standard molecular weight marker (restriction fragment length polymorphism -RFLP) (Sameer AE et al 2009, Shukla P et al 2012). Another alternative method used in order to detect certain specific mutations located in a gene of interest is by an allele specific PCR where specific oligonucleotide primers for hybridization with common or allelic variants are employed in parallel PCR amplification reactions. Only oligonucleotide primers that exactly hybridize to the targeted DNA sequence generate amplified product (De Mare A et al 2010).

A more advanced laboratory method used for detection of specific gene mutations is Real Time-PCR (RT-PCR). Compared to the standard PCR, this is a more advanced molecular method used to multiply and detect (or quantify) simultaneously a specific region of the gene (Edwards KJ et al 2012; Tydén E et al 2014). This procedure corresponds to the general principles of PCR, but a specific feature of this method is that it monitors the progress of a PCR reaction (detection of amplified DNA) in real time. Real-time PCR represent the most useful machine for conducting quantitative nucleic acids analysis for diagnostic and research purposes (Bustin SA, 2004; Bustin SA, 2009). This important technique is an improvement of the original PCR established in the mid-1980s by the scientist Kary Mullis and his collaborators, for which he was awarded the Nobel Prize in Chemistry in 1993 by Royal Swedish Academy of Sciences (Peake I, 1989; Kubista M et al 2006). The PCR machine makes possible amplification of each nucleic acid sequence present in a certain sample (blood, tissue etc.) through a cyclic process that produces a large number of identical copies. Thereafter, these gene copies can be analyzed readily. This technique enabled procedures such as DNA cloning, sequencing and genetic engineering. However, the original PCR method as an analytical technique had significant disadvantages. The process of quantification of DNA sequence was extremely difficult through the use of PCR because this technique produced the same amount of product regardless the amount of DNA template used for this purpose. The development of Real-time PCR in 1992 resolved this handicap of PCR technique (Higuchi, R et al 1992). Real-time PCR is distinguished by the fact that the amount of amplified product may be monitored during the process of reaction by

monitoring the fluorescence of probes that proportionally corresponds to the amount of amplified product. In addition, the number of amplification cycles necessary for obtaining certain amount of amplified product is registered by the Real-time PCR machine. In contrast to the old-fashioned approach, Real-time PCR makes possible to calculate the efficiency of the reaction very precisely. Concretely, it is assumed that during each amplification cycle amplified DNA molecules double. Therefore, this approach allows for the calculation of the number of amplified DNA molecules that were present in the sample at the beginning of the process. Given the details of Real-time PCR mentioned above, such as detection chemistries with high efficiency as well as susceptible instruments and advanced assays that are available currently, the number of molecules of a certain DNA segment within a complex sample becomes possible to be determined with very high sensitivity and accuracy, enough to detect a single DNA molecule. Real-time PCR can be used for different purposes such as single nucleotide polymorphism (SNP) analysis, gene expression analysis, pathogen (bacteria or virus) detection, chromosome aberration analyses and detection of proteins (Kubista M et al 2006; Bustin SA et al 2009).

Genotyping has certain advantages compared to phenotyping methods. A small quantity of blood or specific tissue is needed to perform genotyping and the result is not dependent on the subject's health status or drugs that the subject could use. Besides, when using the RT-PCR technique, the result is obtainedable in a few hours. This analysis is performed once in a lifetime, because genotype remains unchanged during the entire lifetime. To date, a large number of different variants of CYPs have been described, but this biomolecular analyses, for economic reasons, is conducted only for the most frequent mutations present in a population (Puehringer H et al 2010; Dodgen TM et al 2015).

**Table 2. Table presentation of CYP2C9, CYP2C19, CYP3A5 and VKORC1 substrates, inducers, variant alleles and test drugs used for phenotyping**

| Enzyme | Enzyme substrates | Enzyme inhibitors | Enzyme inducers | Most important variant alleles | Test drug for phenotyping | Allele frequency (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Caucasian population | Asian population (China) | African population (African American) |
| **CYP2C9** | diazepam, warfarin, NSAID-s, celecoxib, torasemide, cyclophosphamide, phenytoin, losartan | fluconazole, amiodarone, isoniazid, valproic acid, fenofibrate, and sertraline | barbiturates, carbamazepine, and rifampin-chronic | **2* and **3* | warfarin or phenytoin | 9-16.5% (g, h) | 0-3% | 0.5-3.6% (a) |
| **CYP2C19** | barbiturates, clopidogrel, lansoprazole, diazepam, mephenytoin, imipramine, propranolol, proguanil | omeprazole, chloramphenicol, cimetidine, fluoxetine, and oxcarbamazepine | carbamazepine, rifampicin, pentobarbital, prednisone, | **2*, **3* and **17* | mephenytoin | 13.07-20.02% (i, j, k) | 2-42% (e, f) | 0-19% (b) |
| **CYP3A5** | sirolimus, tacrolimus, imatinib, alfentanil, atorvastatin, clarithromycin, codeine, haloperidol, nifedipine | chlorpheniramine, cocaine, diltiazem, indinavir, lovastatin, methadone, nelfinavir, telithromycin, verapamil | carbamazepine efavirenz glucocorticoids oxcarbazepine phenobarbital phenytoin pioglitazone rifabutin | **3* | midazolam | 85-95.5% (l) | 69-76.7% (c, l) | 19-27.9%, (d, l) |
| **VKORC1** | Vitamin K | warfarin acenocoumarol coumarin | | -1639G>A 1173C>T Lower warfarin dose | warfarin | *TT*-38% *TT* (homozygous for variant allele) (n) | *TT*-91-93% (i) | *TT*-0.9% (m) |

a- Yasuda SU et al 2008; b- Strom CM et al 2012 c- Balram C et al 2003, d- Mirghani RA et al 2006, e- Anichavezhi D et al 2012; f- Scott SA et al 2011, g- Gaikovitch EA et al. 2003, h-Arvanitidis K et al. 2007, i- Yan X et al, 2015 , j- Scordo MG et al 2004, k- Kapedanovska Nestorovska A et al 2015, l- Adler G et al 2009; m- Limdi NA et al, 2008; n, Mandic D et al, 2015;

## 1.5. Cytochrome P 450 2C9 (CYP2C9)

CYP2C9 is an important subcategory within the CYP2C subfamily. Gene coding for CYP2C9 enzyme is located on the long arm of chromosome 10, in the region that contains also genes that encode CYP 2C8, 2C18 and 2C19 enzymes. *CYP2C9* gene is responsible for the 490 amino acids protein coding, weighting of 55.6 kDa (molecular weight) (Van Booven D et al 2010). To date, pharmacogenetic studies have revealed 67 variant alleles, but the most important of them are *CYP2C9*1* (reference haplotype), *CYP2C9*2* and *CYP2C9*3* (www.cypalleles.ki.se/cyp2c9.htm, updated 21-Jan-2016). Each of these variant alleles is characterized by a different catalytic activity. *CYP2C9*2* and *CYP2C9*3* variants yield enzymes with reduced activity, where *CYP2C9*3* variant allele manifest a stronger pharmacokinetic effect than *CYP2C9*2*. These alleles have been found in majority (nearly 85%) of poor metabolizers (García-Martín E et al 2001). Depending on the capacity to metabolize substrates of CYP2C9, individuals can be classified as poor metabolizers (PM), intermediate (IM), or extensive (EM) (Ingelman-Sundberg et al 2007). Poor metabolizers in Caucasians are present with 3-5%. Enzymes coded by CYP2C9 have a key role in the metabolism of nearly one hundred drugs, including warfarin, phenytoin, diazepam, valproic acid, NSAIDs (nonsteroidal anti-inflammatory drugs), celecoxib and so on. Likewise, these enzymes play a substantial role in the metabolism of those drugs with a narrow therapeutic index, such as phenytoin and warfarin (Miao L. et al. 2007). CYP2C9 catalyze nearly 90% of phenytoin metabolism and **2* and **3* haplotypes are responsible for reduced metabolism of phenytoin (Van Booven, D. et al 2010). Given results of Budi et al (2015), *CYP2C9*2* and *CYP2C9*3* genotype determination could be very helpful for improving the safety of antiepileptic drugs such as Valproic acid (VPA) in pediatric patients and subsequently avoiding drug's induced side effects.

*CYP2C9*2* and *CYP2C9*3* variants and mutations in promoter region of VKORC1 (Vitamin K epoxide reductase complex subunit 1) are considered to be responsible for 40-63% of the variability observed in warfarin dosing. Patients carrying these variants are exposed to a greater risk of bleeding in cases of warfarin administration and, consequently, they require lower doses (Kudzi, W. et al 2016).

Besides phenytoin and warfarin guidelines, there are also helpful recommendations concerning CYP2C9 genotypes that have been issued for other medications such as celecoxib, voriconazole, piroxicam, flurbiprofen, and lesinurad [On FDA Biomarker List; voriconazole (VFEND) EMA drug label; Health Canada or Santé Canada].

**Table 3. The main substrates of CYP2C9 enzyme**

| **Drug class** | |
|---|---|
| NSAIDs | ketoprofen, ibuprofen, diclofenac, indomethacin, |
| Antidepressants | fluoxetine, amitriptyline |
| Angiotensin II receptor blockers (ARBs) | irbesartan, losartan |
| Antidiabetic medications | sulfonylureas (glipizide, glibenclamide), rosiglitazone |
| Anticoagulants | S-warfarin |
| Loop diuretics | torasemide |
| Antimicrobics | sulfametoxazole, metronidazole |
| Anticonvulsant | phenytoin |

## 1.6. Cytochrome P450 2C19 (CYP2C19)

Polymorphism of *CYP2C19* is important for pharmacotherapy with barbiturates, clopidogrel, lansoprazole, diazepam, mephenytoin, imipramine, propranolol, and proguanil ("Entrez Gene: *CYP2C19* cytochrome P450, family 2, subfamily C, polypeptide 19", updated on 28-Sep-2015). CYP2C19 activity is strongly linked with bioactivation of the prodrug clopidogrel which is an antiplatelet drug of choice in patients diagnosed with acute coronary syndromes (ACSs), especially those patients undergoing percutaneous coronary intervention (Beitelshees, A. L. et al 2011).

Similar to *CYP2C9*, *CYP2C19* gene is also highly polymorphic. So far, over 30 variant alleles and subvariants have been identified. *CYP2C19*2-*8* variants are non- function alleles that produce an inactive enzyme (Scott SA et al 2012; http://www.cypalleles.ki.se/cyp2c19.htm, updated 1-Oct-2015). The main variant alleles of *CYP2C19* are **2, *3* and **17* allele. Drug metabolizer categories for CYP2C19 are EM, IM, PM, and ultra rapid metabolizers (UM). *CYP2C19*2* and *CYP2C19*3* alleles, account for the majority (95.0%) of the poor metabolizer phenotypes (McGraw J et al 2012; Scott, S. A. et al 2012). Poor metabolizers (e.g.,**2/*2,*2/*3* and **3/*3*), in Caucasians are present with 2-5%, while 20% and 5.2% of Asians and African-Americans respectively belong to this category. The most frequent allele in Caucasians is **2*, and in Asians it is **3* allele (Martis S et al 2013; Fricke-Galindo I et al 2016).

The most important nonfunctional allele of *CYP2C19* is **2* allele (c.681G>A; rs4244285). Presence of *CYP2C19*2* allele is associated with higher risk for adverse cardiovascular events in individuals that are heterozygous or homozygous for this allele (~25–50% of the population) (SA Scott et al 2011). Contrary, *CYP2C19*17* allele (c.-806C>T; rs12248560;) is linked with increased activity as a result of enhanced gene transcription. Subjects that are homozygous for this variant allele (**17/*17*) may be classified as ultrarapid metabolizers. Given some studies, there are some indications that presence of this variant allele generates increased clopidogrel response and platelet inhibition and likely higher risk of bleeding complications (Shuldiner, A. R. et al 2009). In addition, individuals carrying one defective (loss-of-function) allele and a **17* allele represent a heterozygous genotype (e.g.,**2/*17*) and a phenotypic consequences are presently unclear but may be an intermediate form of metabolizers between EM and IM, and possibly may be dependent on the substrate (Li-Wan-Po, A et al 2010). Hicks JK et al 2016, Scott

SA et al 2013 and Moriyama B et al 2016 published interesting and useful recommendations regarding the CYP2C9 genotypes that have been issued for drugs such as clopidogrel, amitriptyline clomipramine, doxepine, imipramine and trimipramine, sertraline, voriconazole and esomeprazole.

**Table 4. The main substrates of CYP2C19 enzyme**

| **Drug class** | |
|---|---|
| Angiotensin II receptor blockers (ARBs) | valsartan, losartan |
| NSAIDs | ketoprofen, ibuprofen, diclofenac, indomethacin, |
| Antidiabetics | glipizide, tolbutamide |
| Antiepileptics | valproic acid, phenytoin, primidone, diazepam, phenobarbitone |
| Proton pump inhibitors | pantoprazole, lansoprazole, omeprazole, rabeprazole |
| Antidepressants | amitriptyline, imipramine, citalopram, escitalopram bupropion, sertraline |
| Other drugs | clopidogrel, warfarin, proguanil, chloramphenicol, propranolol, ritonavir, tolbutamid, cyclophosphamide |

## 1.7. Cytochrome P 450 3A5 (CYP3A5)

The *CYP3A5* gene is one of the isoforms of CYP3A subfamily. CYP3A5, together with the 3A4, 3A7 and 3A43 gene, belong to the CYP genes group, which are located in chromosome 7q21.1 (Gellner K et al 2001). Considering that CYP3A5 belongs to the CYP3A enzymes and primarily is an extrahepatic enzyme, its variant alleles are likely to have an impact on the disposition of several xenobiotics and endogenous compounds in lung, kidney, prostate, breast and white blood cells, and therefore this fact can increase the possibility of developing pathologic conditions in these tissues (Peng et al., 2004). Because of the frequent mutation of adenosine to guanosine at position 6986 within intron 3 (rs776746), CYP3A5 is expressed in only 10% of Caucasians.

The CYP3A5 enzyme contributes in the metabolism of more than half (>50%) of clinically used drugs and a number of procarcinogens and endogenous compounds, as well (Kuehl P et al 2001). The most important substrates of CYP3A5 are benzodiazepines (alprazolam, midazolam etc.), immunosuppressants (cyclosporine and tacrolimus), antibiotics/antivirals (clarithromycin, erythromycin, indinavir etc.), statins (atorvastatin), antipsychotics (quetiapine) and so on. A number of drugs can either induce or inhibit the activity of CYP3A5. The *CYP3A5*1* allele (A at position 6989) encodes the production of normal mRNA, and resultantly it yields a high enzyme expression.

On the other side, the main *CYP3A5* gene variant alleles are **3* (6986A > G; rs776746) and **6* allele (14690G>A; rs10264272). The *CYP3A5*3* allele is characterized by alternative mRNA splicing. For that reason, the intron sequence is incorporated into the mature messenger RNA (mRNA), and, consequently, the premature completion of translation produces nonfunctional protein (Marwa KJ et al 2014). Individuals that are homozygous carriers of *CYP3A5*3* allele (*CYP3A5*3/*3*) are known as non-expressers and lack protein activity compared to individuals that are known as expressers and are carriers of one *CYP3A5*3* allele (*CYP3A5*1*3*) or completely lack this allele (*CYP3A5*1*1*). Carriers of *CYP3A5*3/*3* genotype require lower doses of tacrolimus in order to achieve the target concentration as opposed to expressers (Hesselink, D. A. et al 2003). Additionally, the polymorphism of *CYP3A5* *3 variant allele can also contribute as a risk factor in cancer developing (Sailaja K et al 2010). Additionally, the *CYP3A5*6* variant allele (14690G>A) generates alternate splicing and thereafter protein truncation thus causing a full absence of CYP3A5 enzyme from a specific tissue.

The polymorphism of *CYP3A5* gene is manifested with evident interethnic differences regarding the level of CYP3A5 enzyme (Bains RK et al 2013). Tacrolimus is the backbone therapy for immunosuppression after solid organ and hematopoietic stem cell transplantation. Extensive and intermediate metabolizers of CYP3A5 (*CYP3A5*1/*1* and *CYP3A5*1/*3*) manifest a decreased dose-adjusted trough concentrations of tacrolimus compared to individuals that are CYP3A5 nonexpressers/poor metabolizers (Birdwell, K. A. et al 2015). There are reports confirming the impact of interethnic difference on tacrolimus dosage after conversion from twice-a-day dosing of tacrolimus (known also as Tac-BID) to once per day dosing (also known as Tac-OD) (Glick, L. et al 2014).

In addition to polymorphism's impact, drug–drug interactions and environmental factors are important factors as well, contributing in these interethnic variations. Therefore, CYP3A5 is likely to be an important contributor in intra-ethnic and inter-ethnic differences of CYP3A mediated metabolism (Shirasaka Y et al 2013). Guidelines and recommendations have been issued for CYP3A5 and tacrolimus, (Birdwell KA et al 2015).

**Table 5. CYP3A5 enzyme main substrates**

| **Drug class** | |
|---|---|
| Antidiabetics | nateglinide |
| Antimicrobics and antiprotozoals | clarithromycin, dapsone, telithromycin, quinine |
| Tyrosine kinase inhibitor | imatinib |
| Opioid analgesics | alfentanil, cocaine, codeine, dextromethorphan, fentanyl, methadone, levomethadyl acetate |
| Calcium channel blockers | amlodipine, diltiazem, felodipine, lercanidipine, nifedipine, nisoldipine, nitrendipine, verapamil |
| Benzodiazepines | alprazolam, diazepam, midazolam, triazolam |
| Antidepressants | trazodone, |
| Neuroleptics | haloperidol |
| Antihistamines | astemizole, terfenadine |
| Statins | atorvastatin, cerivastatin, lovastatin, simvastatin, |
| Antivirals | indinavir, nelfinavir, ritonavir, saquinavir |
| Chemotherapeutic and immunomodulatory drugs | cyclosporine, paclitaxel, docetaxel, tamoxifen, irinotecan, vincristine, sirolimus, tacrolimus |
| Other drugs | aripiprazole, buspirone, caffeine, cilostazol, cisapride, domperidone, eplerenone, estradiol, finasteride, hydrocortisone, lidocaine, ondansetron, pimozide, progesterone, propranolol, salmeterol, sildenafil, testosterone, zaleplon, zolpidem |

## 1.8. VKORC1

Apart from CYP2C9 and CYP2C19, warfarin metabolism is also influenced by vitamin K epoxide reductase complex 1 (*VKORC1*) that encodes the warfarin target protein (Wijnen PA et al. 2010; Suriapranata IM et al. 2011). Furthermore, this enzymatic complex (Rost S et al. 2004) recycles the reduced vitamin K, which is necessary for the posttranslational carboxylation of vitamin K-dependent clotting factors, such as prothrombin, FVII, FIX, and FX. The main variants of VKORC1 are *VKORC1 [G3673A; (rs9923231)]*, *VKORC1 [C6484T (rs9934438)]* and *VKORC1 [G9041A (rs7294)]* (Owen RP et al 2010). Moreover, there is a strong linkage disequilibrium between the *VKORC1 –1639G>A* and *1173C>T* polymorphisms (Seip RL et al. 2010). These two polymorphisms manifest a direct impact on the effectiveness (potency) of a given dose of coumarin anticoagulant and on its response (D'Andrea G et al. 2005; Zhu Y et al. 2007). Studies on a Caucasian population have proved that in the cases of thromboembolic disorders, the carriers of the *VKORC1 –1639 AA* genotype require a significantly lower daily dose of warfarin compared to carriers of the GA or GG genotypes (Molden E et al. 2010). As for the *VKORC1 –1639 AA* genotype subjects, individuals with the TT genotype of *VKORC1 (1173C>T)* also require a lower warfarin maintenance dose compared to *CC* genotype (Yan X et al 2015). Clinical Pharmacogenetics Implementation Consortium (CPIC) published updated guidelines for pharmacogenetics-guided warfarin dosing which are valid for both pediatric and adult patients (Johnson JA et al 2017). Pharmacogenetics-guided dosing has been published for other anticoagulant such as acenocoumarol and phenprocoumon (Swen JJ et al 2011).

**Table 6. Genes and variant alleles of cytochrome P450 and VKORC1 analyzed in this study**

| Variant allele | Single nucleotide base change | Enzyme activity |
|---|---|---|
| *CYP 2C9 *2* | c.430C> T | Decreased |
| *CYP 2C9 *3* | c.1075A >C | Decreased |
| *CYP 2C19 *2* | c.681G>A | None |
| *CYP 2C19 *17* | c.-806C>T | Increased |
| *CYP3A5 *3* | g.6986A > G | none |
| *VKORC1* | 1173C>T | Decreased |

# 2. HYPOTHESIS

Research conducted in other populations has shown that inter-ethnic differences of pharmacogenetic profile have a direct impact on pharmacotherapy. Thereby, we supposed that in Kosovo's population there are certain specificities of pharmacogenetic profile that deserve a closer examination.

# 3. AIMS AND PURPOSE OF THE RESEARCH

According to the present knowledge, the response to the clopidogrel, warfarin, NSAIDs, tacrolimus and imatinib pharmacotherapy is determined by a complex system of interactions at different levels; therefore, we supposed that predicting the CYPs and VKORC1 polymorphisms' value is important. In this context, we aimed to assign the frequency of major variant alleles of *CYP2C9, CYP2C19, CYP3A5* and *VKORC1* gene in order to use these data for identification of individuals of risk group, as well as mark them as potential molecular markers in pharmacodiagnostics and clinical practice.

## 3.1. GENERAL AIM

The main purpose of this study was to conduct a pharmacogenetic screening in Kosovo's healthy population. Information obtained from this research was used to compare the allele frequency in Kosovo's population with that of other ethnic groups.

## 3.2. SPECIFIC AIM

The more specific aim of this research was to investigate the frequency of *CYP2C9, CYP2C19, CYP3A5* and *VKORC1* polymorphisms in Kosovo's healthy population.

# 4. MATERIALS AND METHODOLOGY

## 4.1 Research participants

All participants have been enrolled provided that they sign a consent and information form. Research has commenced upon issuance of permission by the Ethics Committee of the University Clinical Center of Kosovo in Prishtina and the Faculty of Pharmacy in Skopje where all molecular analyses were performed.

### 4.1.1. Healthy subjects

The main criterion of inclusion in the research for this group of participants has been the absence of any mental illness or any history of serious physical illness based on individual's personal history. The distribution of allelic variants of *CYP2C9, CYP2C19, CYP3A5* and *VKORC1* in Kosovo's population has been analyzed in 234 people aged between 18 and 65 years. Research has been carried out in healthy persons without blood relationship between them, representing a mixed population from all the parts of Kosovo. The gender ratio of participants in the research was nearly equal between women and men (116:118).

Research participants included mainly academic and non-academic staff of the Faculty of Medicine in Prishtina, medical and non-medical staff of the University Clinical Center of Kosovo, as well as medical and non-medical staff of the Main Family Medicine Center in Prishtina.

## 4.2 Material

### Analytical samples

In the process of DNA extraction (isolation), 5 ml of fresh blood together with the anticoagulant ethylene diamine tetra-acetic acid (EDTA) has been used from each research participant. Blood samples have been stored at -4°C for 3 days (maximum), until the analyses have been performed.

## 4.3 DNA analyses

### 4.3.1 DNA isolation procedure

Genomic DNA has been extracted from whole blood, using QIAGEN DNA extraction kit and the procedure recommended by the manufacturer (QIAGEN AS, Oslo, Norway). This method, which is based on cell lysis, enzymatic and chemical extraction, has been used in order to remove cellular proteins, ribonucleic acid (RNA), and other macromolecules. This has been followed by DNA precipitation in absolute alcohol.

**Laboratory equipments**:

| | | |
|---|---|---|
| 1. | Microcentrifuge tube | Eppendorf AG, Germany |
| 2. | QIAamp Mini spin column (2ml) | Qiagen GmbH, Germany |
| 3. | Collection tubes (2ml) | Qiagen GmbH, Germany |
| 4. | Refrigerated Centrifuge | Centurion Scientific, UK |
| 5. | Dry block thermostat at 56º C | BioSan, Latvia |
| 6. | Vortexer 2x3 | UNI EQUIP, Germany |
| 7. | Microcentrifuge (with rotor for 2ml tubes) | Mini spin plus, Germany |
| 8. | Automatic Pipettes Eppendorf research | Thermo Scientific, USA |
| 9. | Pipet tips with aerosol barrier Top- Line | Germany |

**(**QIAamp DNA mini handbook, page no 16)

**Materials**

| | |
|---|---|
| QIAGEN Proteinase K (store at 2-8°C or -20°C), | Qiagen GmbH, Germany |
| As indicated on the label, when using the QIAamp DNA blood mini kit (250), 5.5 ml of protease solvent should be pipetted into the vial containing lyophilized QIAGEN protease. | |
| Buffer AL | GmbH, Germany |
| Ethanol (96-100%) | Alkaloid, Macedonia |
| Buffer AW1 | GmbH, Germany |
| Buffer AW2 | GmbH, Germany |
| Buffer AE | GmbH, Germany |

**(**QIAamp DNA mini handbook, page no 27)

## Procedure

1. 20 µl of QIAGEN Protease (or proteinase K) should be pipetted into the bottom of the 1.5 ml microcentrifuge tube.
2. Thereafter, 200 µl of the sample (participant's blood) should be added into the microcentrifuge tube.
3. 200 µl of Buffer AL should be pipetted into the 1.5 ml microcentrifuge tube together with participant's sample and QIAGEN Protease. After that, this mixture should be mixed by pulse-vortexer for 15 seconds.
4. Mixture should be incubated at 56°C for 10-20 minutes.
5. The 1.5 ml microcentrifuge tube should be briefly centrifuged in order to remove drops from the inside of the lid.
6. 200 µl ethanol (96-100%) should be pipetted to the sample, and mixed again by pulse-vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge tube should be centrifuged briefly to remove drops from the inside of the lid.
7. In case the volume is more than 200 µl, the amount of the ethanol should be increased proportionally. Example: 300 µl of ethanol should be pipetted into 300 µl of sample.
8. The mixture obtained from step six carefully should be applied to the QIAamp Mini spin column (2ml), but being careful not to wet the rim. After that, the cap of the Mini spin column (QIAGEN) should be closed and centrifuged at 8000 rpm for 1 minute (60 seconds). This column should be placed in a clean 2ml collection tube to remove the filtrate from the tube.
9. The QIAamp Mini spin column should be opened cautiously and after that add 500 µl Buffer AW1 carefully without wetting the rim. The cap of the column should be closed and centrifuged at 8000 rpm for 1 minute. QIAamp Mini spin column should be placed in a clean 2 ml collection tube, while discardind the filtrate from the collection tube.
10. The QIAamp Mini spin column should be opened cautiously and add 500 µl Buffer AW2 without wetting the rim. After closing the cap, it should be centrifuged at full speed (14000 rpm) for 3 min.
11. Recomended. The QIAamp Mini spin column (not provided) should be placed in a new 2 ml collection tube, and the filtarte from the old collection tube discarded. Centrifuge the

column at full speed for 1 minute. This procedure makes possible to avoid the eventual carryover of Buffer AW2.

12. The QIAamp Mini spin column (not provided) should be placed in a clean 1.5 ml microcentrifuge tube and then discard the filtrate from the collection tube. The QIAamp Mini spin column should be opened cautiously and add either 200 µl Buffer AE or distillated water. Thereafter it should undergo incubation at room temperature (15-25 °C) for 1 minute, and then centrifuged at 8000 rpm for 1 minute.

### 4.3.2 Determination of DNA yield, concentration and purity

DNA yield (from the concentration of DNA in the eluate) was determined diluting the solution in the ratio 1:100 with TE buffer and absorbance (A) has been measured spectrophotometrically at wavelength 260 nm:

$C_{DNA}$ = dilution x F x A = 100 x 50 x A

Purity was determined calculating the ratio of absorbance at 260 ηm to absorbance at 280 ηm. Pure DNA has an $A_{260}/A_{280}$ ratio of 1.7-1.9.

### 4.3.3 Testing of the quality of isolated DNA

The quality of isolated DNA has been tested by electrophoresis in 0.3% agarose gel with ethidium bromide.

## Materials:

| | |
|---|---|
| 1. 0.3% agarose gel | |
| Agarose | 0.6 (BioRad, USA) |
| Distilled water up to | 200 ml (Alkaloid, Macedonia) |
| 2. Ethidium bromide | 10 mg/ml (Sigma Aldrich Chemie, USA) |
| 3. 50x TEA buffer | |
| Tris or Tris(hydroxymethyl)aminomethane | 242 g (Sigma Life Science, USA) |
| EDTA [500mM (pH 8.0) solution] | 100 mL (Sigma Aldrich, USA) |
| Acetic acid | 57,1 mL (Alkaloid, Macedonia) |
| Distilled water up to | 1000 ml (Alkaloid, Macedonia) |
| 4. 1x TEA- buffer: 20 ml 50x TEA+ 200 µL Ethidium-bromide (10 µL/mL), add 1000ml water | |
| 5. The buffer for the sample application on the gel | |
| Bromophenol blue 1 % | 1 mL (Merck, Germany) |
| Xylene cyanol 1% | 1mL (Sigma Aldrich, USA) |
| Glycerol 50 % | 5 mL (Merck, Germany) |
| 50x TEA- buffer 2 % | 190 µL (Merck, Germany) |
| Distilled water | 2,75 mL (Alkaloid, Macedonia) |

## Procedure

1. First, we should add 1 g of agarose in 200 ml of distilled water.
2. In the second step, in the microwave, the solution should be heated to boiling in order to dissolve the agarose. Subsequently, 400 µL 50x TEA- buffer and 2.8 ml of ethidiume bromide should be added.
3. Samples containing 9µL of DNA solution in TE buffer and 1µL buffer with color should be applied on the molded and prepared gel. Terms of electrophoresis: 40 V.
4. The conduction of DNA fragments detection is performed under a fluorescent lamp and the documentation of the results is carried out in a photography Polaroid film 667.

## 4.4. Genotyping methods

### Materials used for DNA molecular analyses:

| | |
|---|---|
| TaqMan Universal PCR Master Mix (5x5mL, 1000 rnx) | Applied Biosystems, USA (Thermo Fisher Scientific) |
| KIT, TAQMAN DRUG METABOLISM | Applied Biosystems, USA (Thermo Fisher Scientific) |
| Multiplate 96-Well PCR Plates, natural, 25 | Applied Biosystems, USA (Thermo Fisher Scientific) |
| Optical Flat 8-Cap Strips, for 0.2 ml tubes and plates, ultraclear, 120 units | Applied Biosystems, USA (Thermo Fisher Scientific) |
| Primers 25 nmol | Invitrogen, USA (Thermo Fisher Scientific) |
| QIAGEN Protease | QIAGEN, Germany |

### Equipment used for DNA molecular analyses

| | |
|---|---|
| Stratagene™ Mx3005P qPCR Instrument | Agilent Technologies, USA |
| Centrifuge | Beckman, Eppendorf, Germany |
| Spectrophotometer NanoDrop 2000 | Thermo Scientific, USA |
| Erlenmeyer flask | Isolab, Germany |
| Microwave oven | Gorenje, Slovenia |
| Electrophoresis system | Mini-Sub® Cell GT, USA |
| Molecular imager VersaDoc MP 4000 system | Bio-Rad Laboratories, USA |
| Cleanroom Bench BIOSAFE | Ehret, Germany |

### 4.4.1 Real time-PCR

Genotyping of *CYP2C9, CYP2C19, CYP3A5* and *VKORC1* has been carried out using ABI TaqMan assays on Stratagene RealTime PCR machine. The single nucleotide polymorphisms (SNPs) in *CYP2C9, CYP2C19, CYP3A5* and *VKORC1* genes has been analyzed by allelic discrimination TaqMan assay (Applied Biosystems, Foster City, CA, USA) using the TaqMan DME genotyping assay according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The basic principle of PCR is the exponential multiplication of genes of interest within the DNA molecules. In order to multiply certain DNA segment, 2 µl of DNA together with 10,5 µl PCR mix {3,625 µl $H_2O$, 6,25 µl master-mix [DNA polymerase, deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP) ] and 0.625 µl of gene specific primer} underwent incubation according to specific conditions. The PCR procedure contains three main steps during each cycle:

**Step 1** (Denaturation of DNA): Incubation in high temperature (till 95°C) is used to "melt" DNA from double-stranded into single strands as well as to lose secondary structure in single strands of sample's DNA. The maximum temperature during reaction's cycle in which DNA polymerase is still active is 95°C. If the guanine-cytosine content (GC content) of template is high, we might have an increase in denaturation time.

**Step 2** (Annealing): During this step of reaction, at 54°C, hybridisation between complementary segment of single stranded of DNA and specific primer occurs.

**Step 3** (Extension): With contribution of the DNA polymerase, at 72°C, extension of primer occurs at rates of up to 100 nucleotide bases/sec and consequently is synthesized complementary stranded of DNA. In cases when an amplicon of Real-time PCR reaction is small, this phase of reaction at 60°C, usually is combined with the annealing phase of reaction.

In the next cycle of reaction, the newly created DNA molecules become "pattern". In order to obtain sufficient material for further genetic analysis, multiplication was performed through 50 cycles.

1. During cooling cycles, ahead to the extension phase, hybridisation of TaqMan probe to its complementary segment in the amplicon of PCR occurs.

2. Throughout polymerisation, when there is an encountering between Taq polymerase and TaqMan probe, 5’ to 3’ exonuclease activity results in the digestion of the reaction probe.

3. Hence, this process separates the donor (FAM) from the acceptor (TAMRA) of fluorescence and subsequently excitation at 495 nm is followed by the fluorescence emission by FAM at wavelength of 525nm.

4. Considering that in this momentum two fluorescent dyes are in a considerable distance from each other, energy transfer from FAM to TAMRA dye cannot happen.

5. Hence, when TaqMan probe has been digested, fluorescence of the TAMRA dye does not occur.

6. In general, the increase of fluorescence emission at 525nm wavelength, on the one hand, and a decrease of fluorescence emission at 585 nm wavelength, on the other, is an indicator of a positive PCR reaction.

In each PCR reaction, genomic DNA, specific primers, DNA polymerase concentration, $Mg^{++}$ (magnesium ions), as well as time and temperature for each step of the reaction, depends on the analytical system and are tested for every segment of genomic DNA.

## Chemicals and equipment used for genotyping

1) deoxyribonucleotide triphosphates (dATP, dGTP, dCTP and dTTP)

- 6,5 µl PCR Master Mix, 2X (50 units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µMdATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM $MgCl_2$.

2) 3,625 µl distillated $H_2O$

3) 0.625 µl of gene specific primer

4) 2 µl of DNA

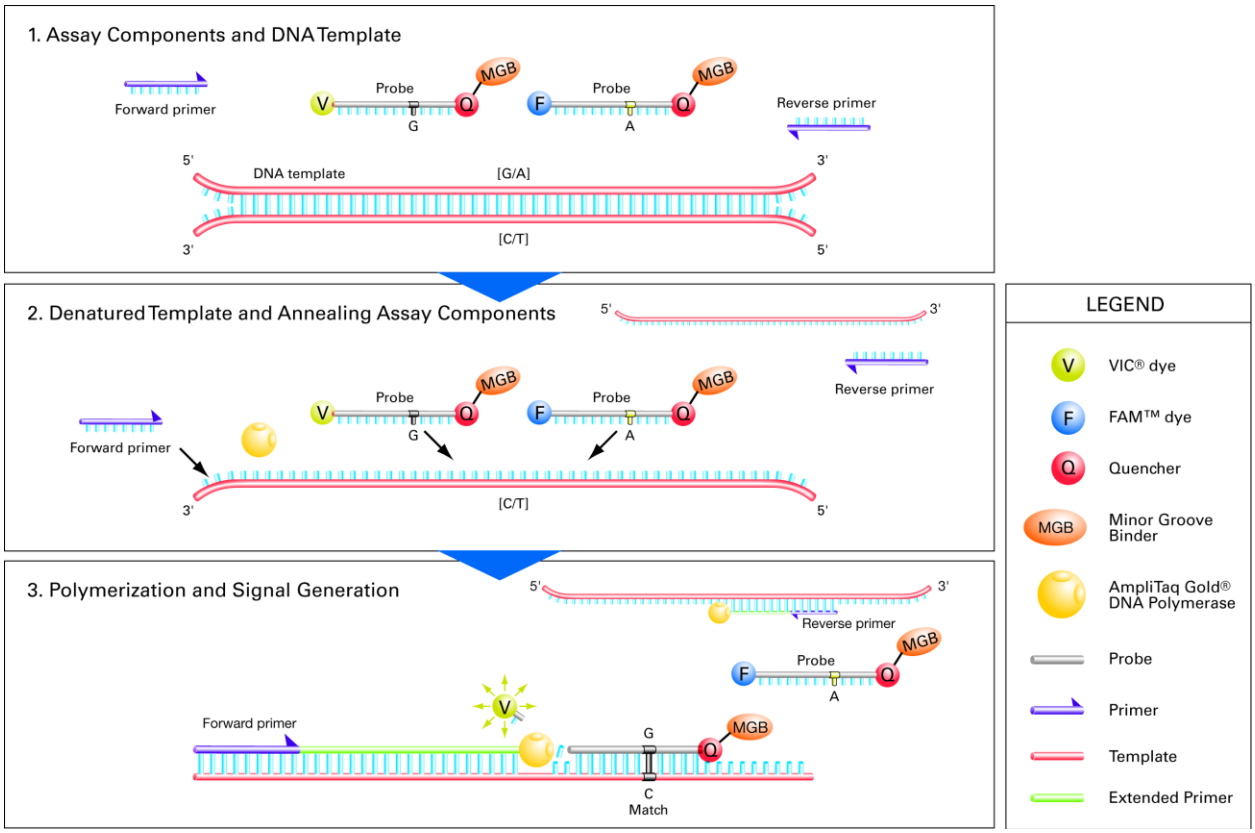


**Figure 1.** Schematic description of a TaqMan® Drug Metabolism Genotyping Assay (Reference Guide, Applied Biosystems)

### 4.4.1.1 *CYP2C9* genotyping

#### 4.4.1.1.1 Genotyping for the **2* variant allele of *CYP2C9*

For multiplication of the *CYP2C9*2* variant, 2 µl of participant's genomic DNA together with 10,5 µl PCR mix [3.625 µl $H_2O$, 6.25 µl master-mix (DNA polymerase, dATP, dTTP, dGTP, dCTP) and 0.625 µl of gene specific primer] underwent incubation according to specific conditions.

Real Time PCR thermal conditions for CYP2C9*2 variant were:

2 minutes (50°C)

10 minutes (95°C),

15sec (92°C) } 50 cycles

60sec (60°C) }

**Table 7. *CYP2C9*2* variant**

***CYP2C9*2*** (**2* allele)
**SNP ID**: rs1799853
**Assay code**: C_25625805_10
**Gene:** *CYP2C9*
**Gene Name:** cytochrome P450, family 2, subfamily C, polypeptide 9
**Set Membership:** > HapMap > DME > Validated > Inventoried
**Chromosome Location:** Chr.10: 96702047 - 96702047 on NCBI Build 37
**Polymorphism:** C/T, Transition Substitution
Context sequence [VIC/FAM] is C/**T**
GATGGGGAAGAGGAGCATTGAGGAC**[C/T]**GTGTTCAAGAGGAAGCCCGCTGCCT
***T*-allele** frequency in Caucasians is **0.17** (or 17%)

***C/C***- Homozygous for normal allele
***C/T***- Heterozygous for variant allele
***T/T***- Homozygous for variant allele

**Total** number of samples = 234
***C/C*=**160 Samples
***C/T***=66 Samples (66 variant alleles)
***T/T***= 8 Samples (16 variant alleles)

***T*** allele frequency in this study = 82/468 = **17,52%**
(https://www.thermofisher.com/order/genome-database/browse/snp/keyword/C__25625805_10)_

**Real-time PCR result for *CY2C9*1*2***

(heterozygous for variant*2)

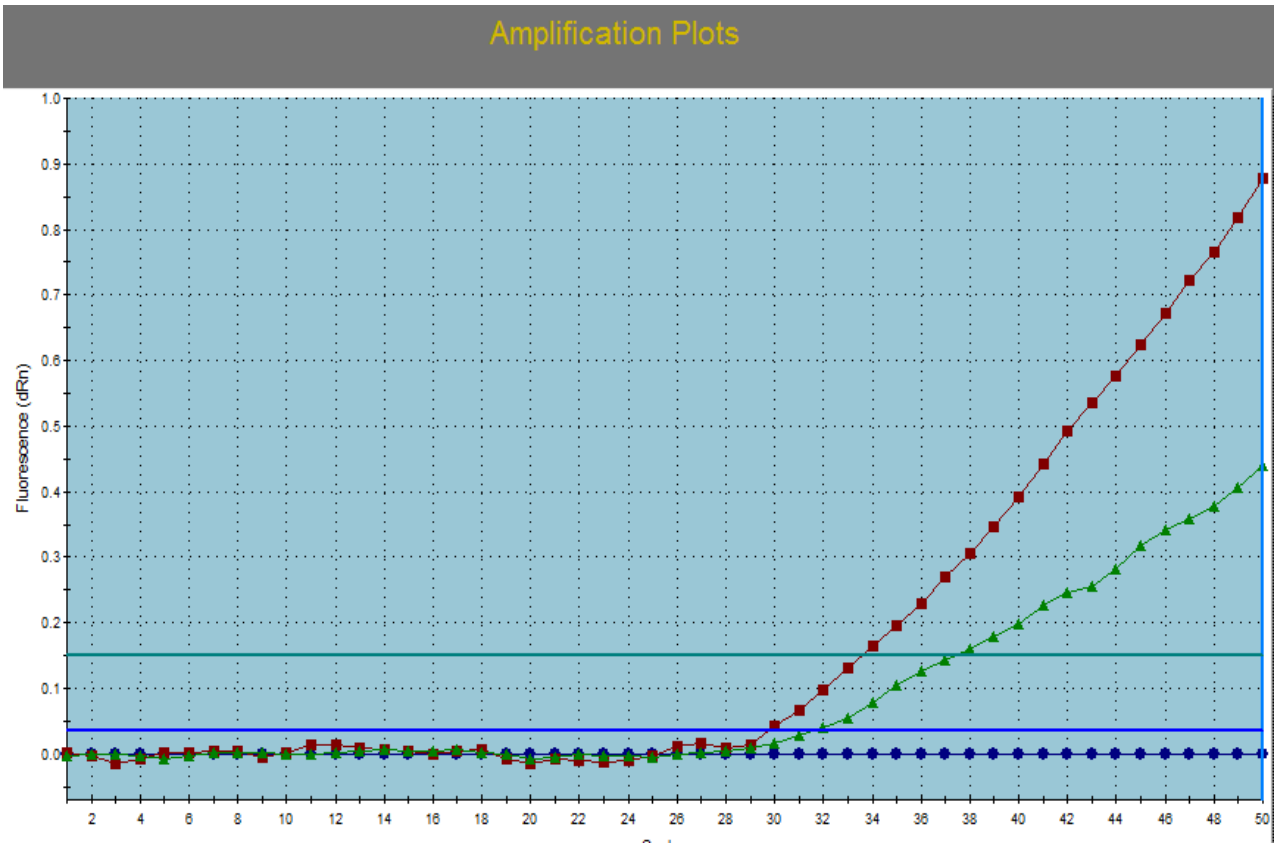


**Figure 2.**

**Real-time PCR result for *CY2C9*2*2***

(homozygous for variant *2)

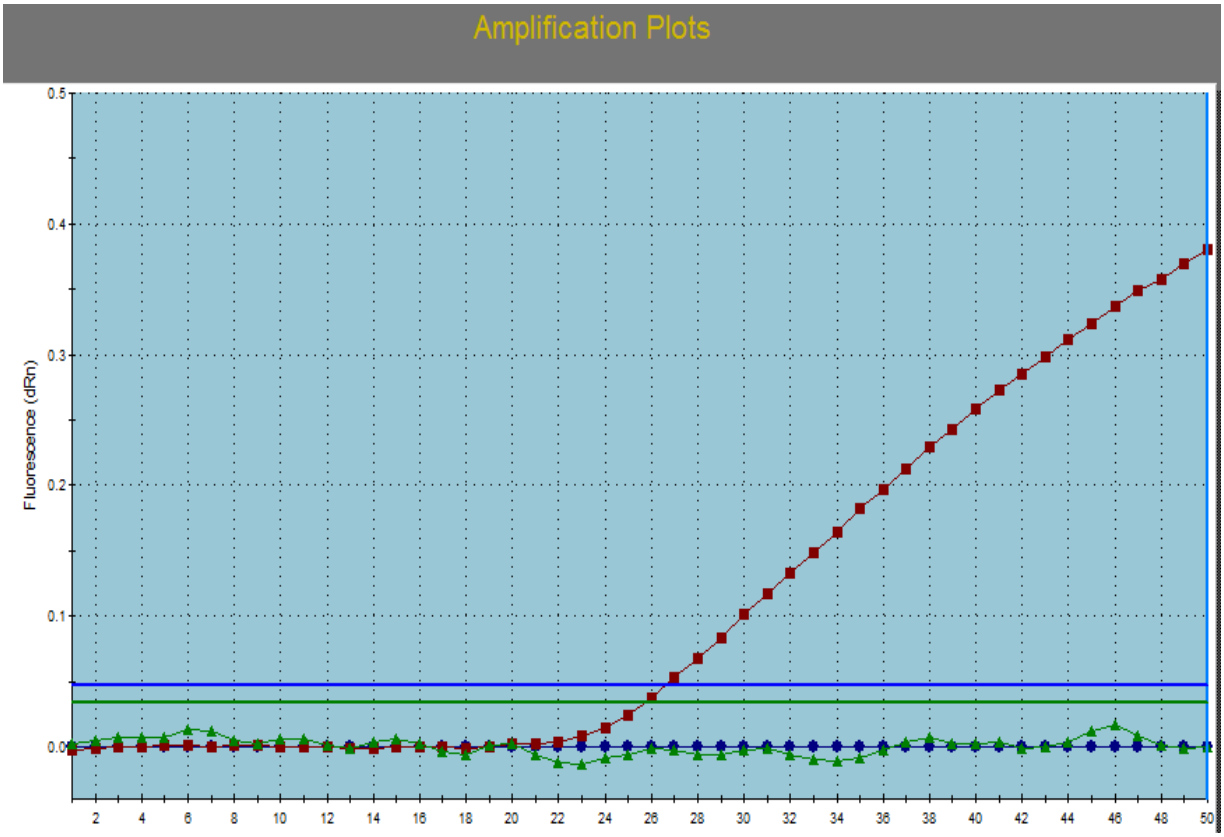


**Figure 3.**

#### 4.4.1.1.2 Genotyping for the **3* variant allele of *CYP2C9*

In order to multiply the *CYP2C9*3* variant, 10,5 µl PCR mix [3.625 µl $H_2O$, 6.25 µl master-mix (DNA polymerase, dATP, dTTP, dGTP, dCTP) and 0.625 µl of gene specific primer] together with 2 µl of participant's DNA underwent incubation according to specific conditions.

Thermal conditions of Real Time PCR for variant **3* of *CYP2C9* were:

2 minutes (50°C)

10 minutes (95°C),

15sec (92°C) } 50 cycles

60sec (60°C) }

**Table 8. *CYP2C9*3* variant**

**SNP ID**: rs1057910
**Assay code**: C_27104892_10
**Gene:** *CYP2C9*
**Gene Name:** cytochrome P450, family 2, subfamily C, polypeptide 9
**Set Membership:** > HapMap > DME > Validated > Inventoried
**Chromosome Location:** Chr.10: 96741053 - 96741053 on NCBI Build 37
**Polymorphism:** C/A, Transversion Substitution
**Context Sequence** [VIC/FAM]: **C**/A
TGTGGTGCACGAGGTCCAGAGATAC**[C/A]**TTGACCTTCTCCCCACCAGCCTGCC
***C*-**allele frequency in Caucasians is **0.1 (or 10%)**

***A/A*-** Homozygous for normal allele
***C/A*-** Heterozygous for variant allele
***C/C*-** Homozygous for variant allele

**Total** number of samples = 234
***A/A*=** 187 Samples
***C/A*=** 43 Samples (43 variant alleles)
***C/C*=** 4 Samples (8 variant alleles)

***C*** allele frequency in this study =51/468=**10,89%**
(https://www.thermofisher.com/order/genome-database/browse/snp/keyword/C_27104892_10)

**Real-time PCR result for *CY2C9*1*3***

(heterozygous for variant *3)

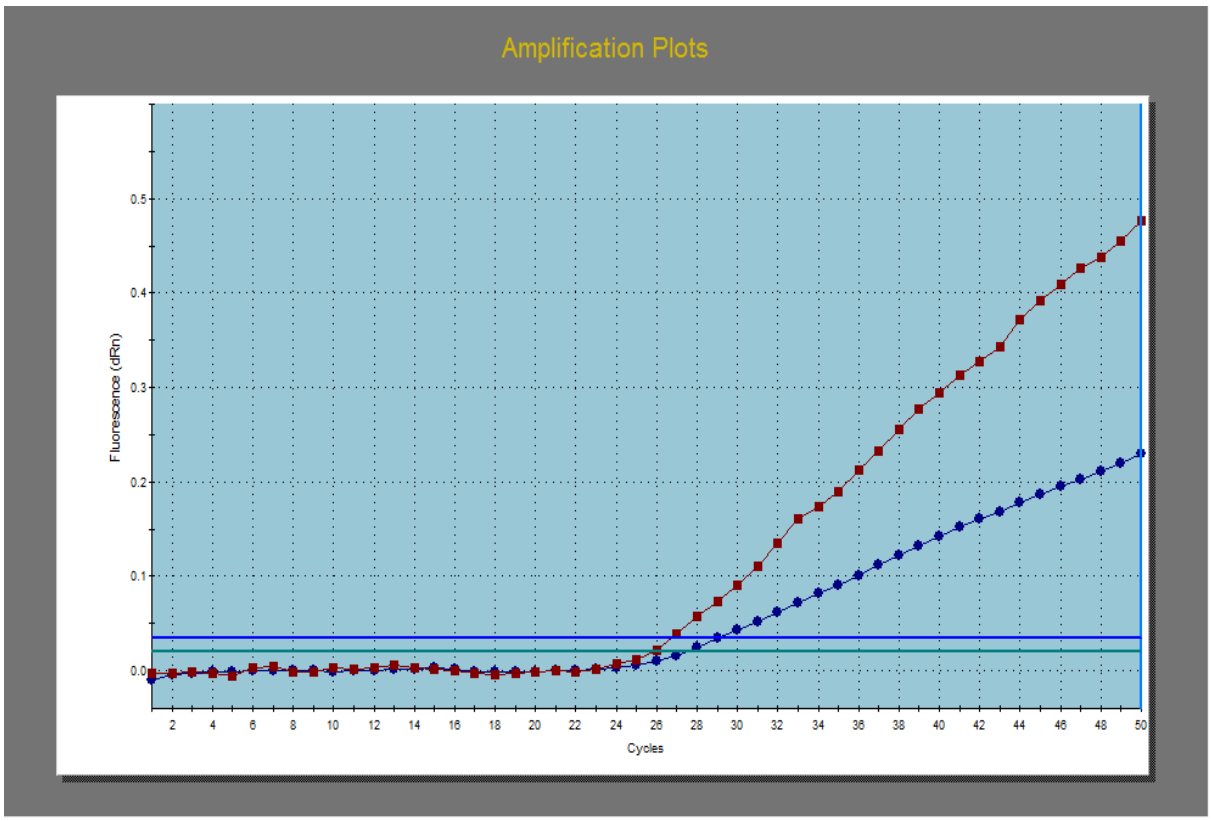


**Figure 4.**

**Real-time PCR result for *CY2C9*3*3***

(homozygous for variant **3*)

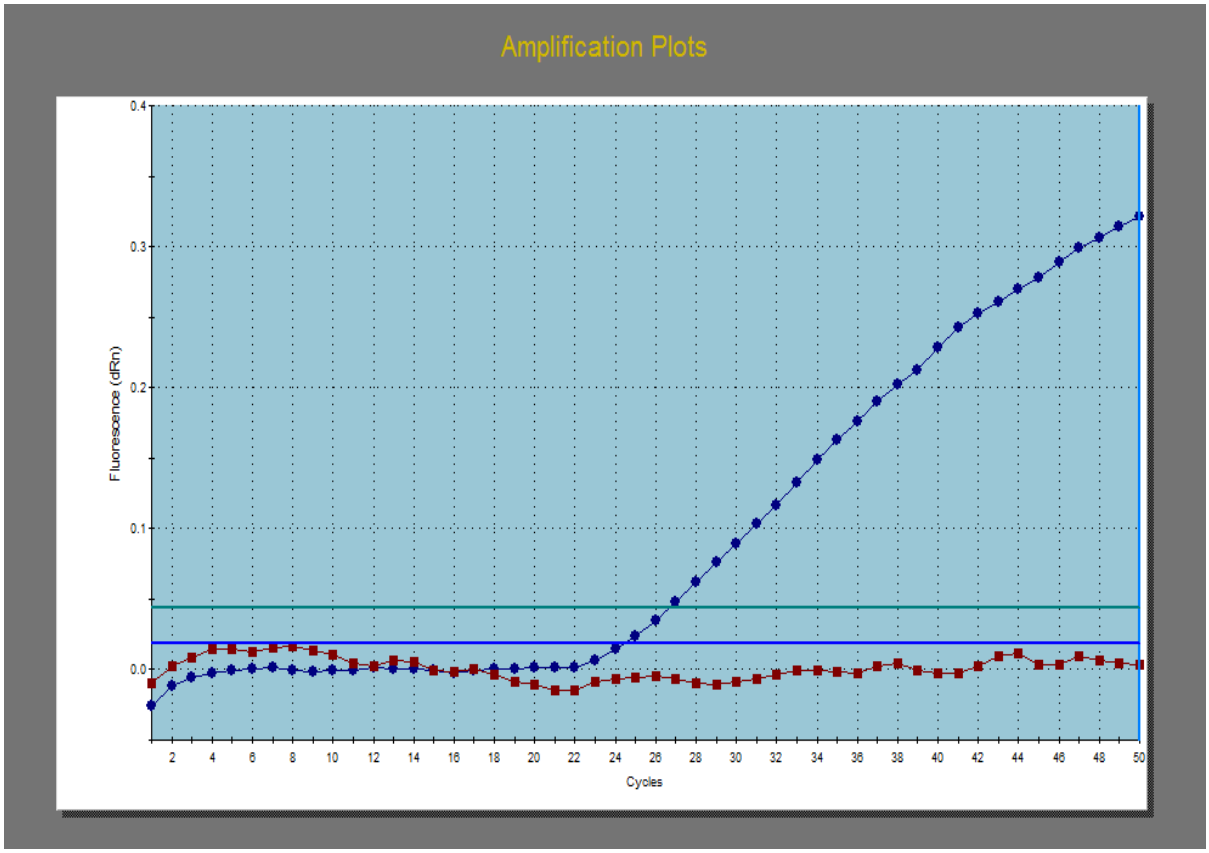


**Figure 5.**

### 4.4.1.2 *CYP2C19* genotyping

#### 4.4.1.2.1 Genotyping for the **2* variant allele of *CYP2C19*

To multiply the **2* variant of *CYP2C19*, 10,5 µl PCR mix [3.625 µl $H_2O$, 6.25 µl master-mix (DNA polymerase, dATP, dTTP, dGTP, dCTP) and 0.625 µl of gene specific primer] together with 2 µl of participant's DNA underwent incubation according to specific conditions.

Real Time PCR thermal conditions for variant *2 of CYP2C19 were:

2 minutes (50°C)

10 minutes (95°C),

15sec (92°C) } 50 cycles

60sec (60°C) }

**Table 9. *CYP2C19*2*** variant

**SNP ID:** rs4244285
**Assay code:** C_25986767_70
**Gene:** *CYP2C19*
**Gene Name:** cytochrome P450, family 2, subfamily C, polypeptide 19
**Set Membership:** > HapMap > DME > Validated > Inventoried
**Chromosome Location**: Chr.10: 96541616 - 96541616 on NCBI Build 37
**Polymorphism:** A/G, Transition Substitution
**Context sequence [VIC/FAM] is A/**G
TTCCCACTATCATTGATTATTTCCC**[A/G]**GGAACCCATAACAAATTACTTAAAA
***A*-allele frequency in Caucasians is 0.14 (or 14%)**

***G/G*-**Homozygous for normal allele
***A/G*-**Heterozygous
***A/A*-**Homozygous for variant allele

**Total** number of samples = 234
***G/G*=**178 Samples
***A/G*=**51 Samples (51 variant alleles)
***A/A*=** 5 Samples (10 variant alleles)

***A*** allele frequency in this study **=61/468=13,03%**
(https://www.thermofisher.com/order/genome-database/browse/snp/keyword/C_25986767_70)

**Real-time PCR result for *CY2C19*1*2***

(heterozygous for variant **2*)

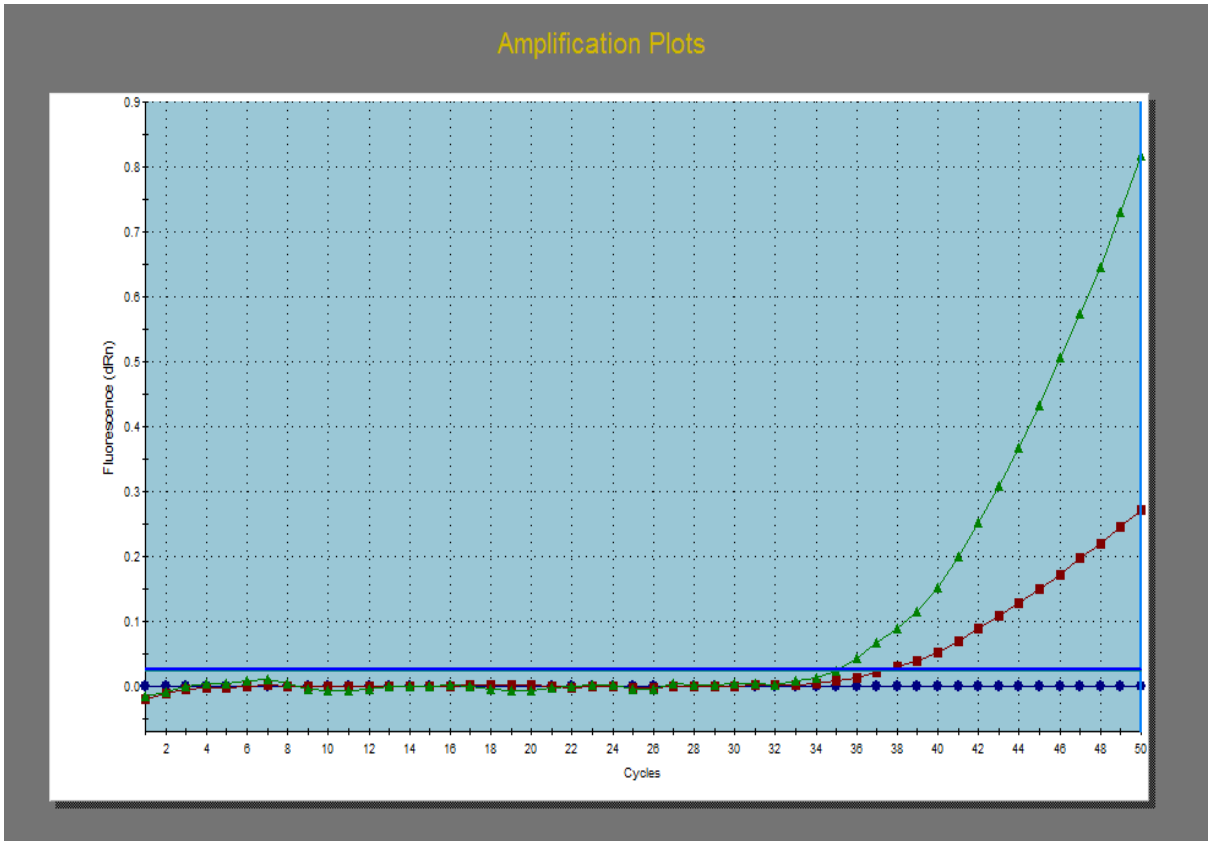


**Figure 6.**

**Real-time PCR result for *CY2C19*2*2***

(homozygous for variant *2)

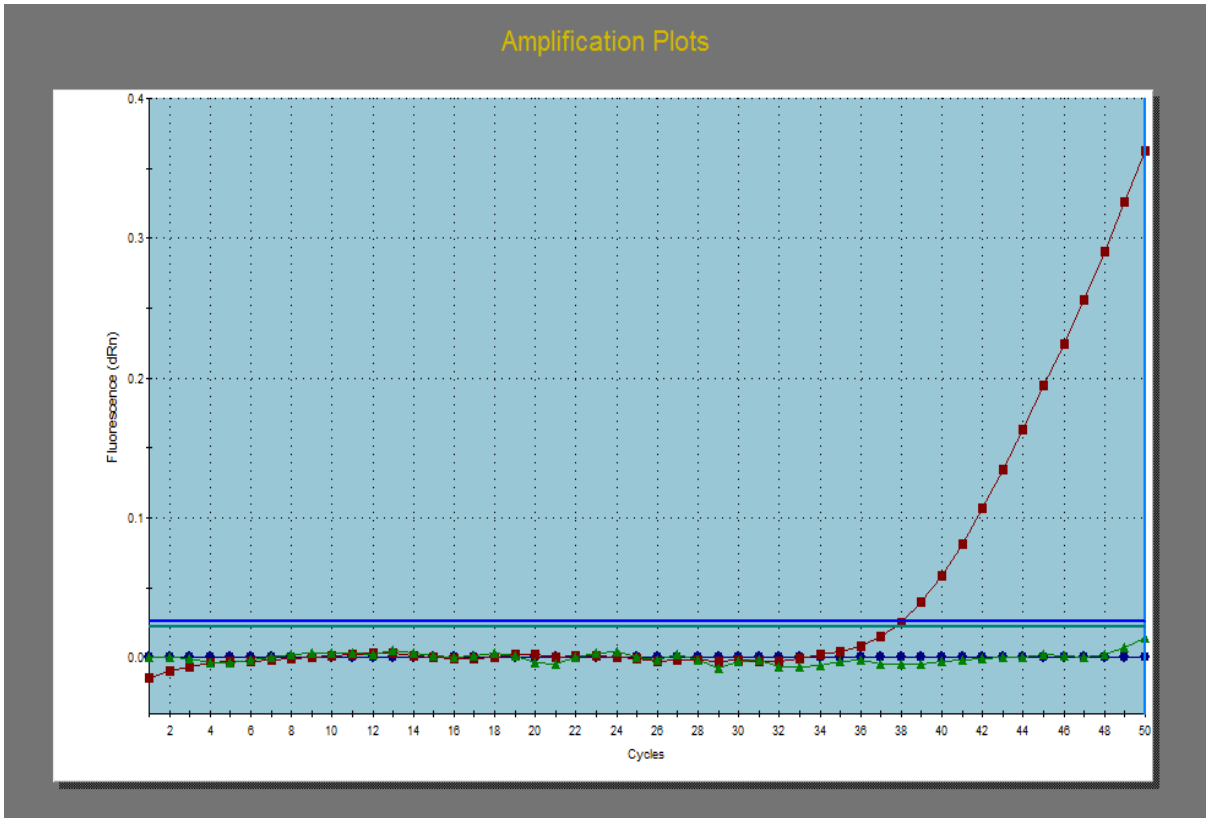


**Figure 7.**

#### 4.4.1.2.2 Genotyping for the **17* variant allele of *CYP2C19*

For multiplication of the **17* variant of *CYP2C19*, 2 µl of participant's DNA with 10,5 µl PCR mix [3.625 µl $H_2O$, 6.25 µl master-mix (DNA polymerase, dATP, dTTP, dGTP, dCTP) and 0.625 µl of gene specific primer] underwent incubation according to specific conditions.

Real Time PCR thermal conditions for variant *17 of CYP2C9 were:

2 minutes (50°C)

10 minutes (95°C),

15sec (92°C) } 50 cycles

60sec (60°C) }

**Table 10. *CYP2C19* 17* variant**

**SNP ID:** rs12248560
**Assay code:** C_469857_10
**Gene:** *CYP2C19*
**Gene Name:** cytochrome P450, family 2, subfamily C, polypeptide 19
**Set Membership:** > HapMap > DME > Validated > Inventoried
**Chromosome Location**: Chr.10: 96521657 - 96521657 on NCBI Build 37
**Polymorphism:** C/T, Transition Substitution
**Context sequence [VIC/FAM] is** C/**T**
AAATTTGTGTCTTCTGTTCTCAAAG**[C/T]**ATCTCTGATGTAAGAGATAATGCGC
***T*-allele frequency in Caucasians is 0.17 (or 17%)**

***C/C***-Homozygous for normal allele
***C/T***-Heterozygous
***T/T***-Homozygous for variant allele

**Total** number of samples = 234
***C/C*=** 155 Samples
***C/T*=** 69 Samples (67 variant alleles)
***T/T*=** 10 Samples (20 variant alleles)

***T*** allele frequency in this study **= 89/468 = 19,01%**
(https://www.thermofisher.com/order/genome-database/browse/snp/keyword/C_469857_10)

**Real-time PCR result for *CY2C19*2*17***

(heterozygous for variant **17*)

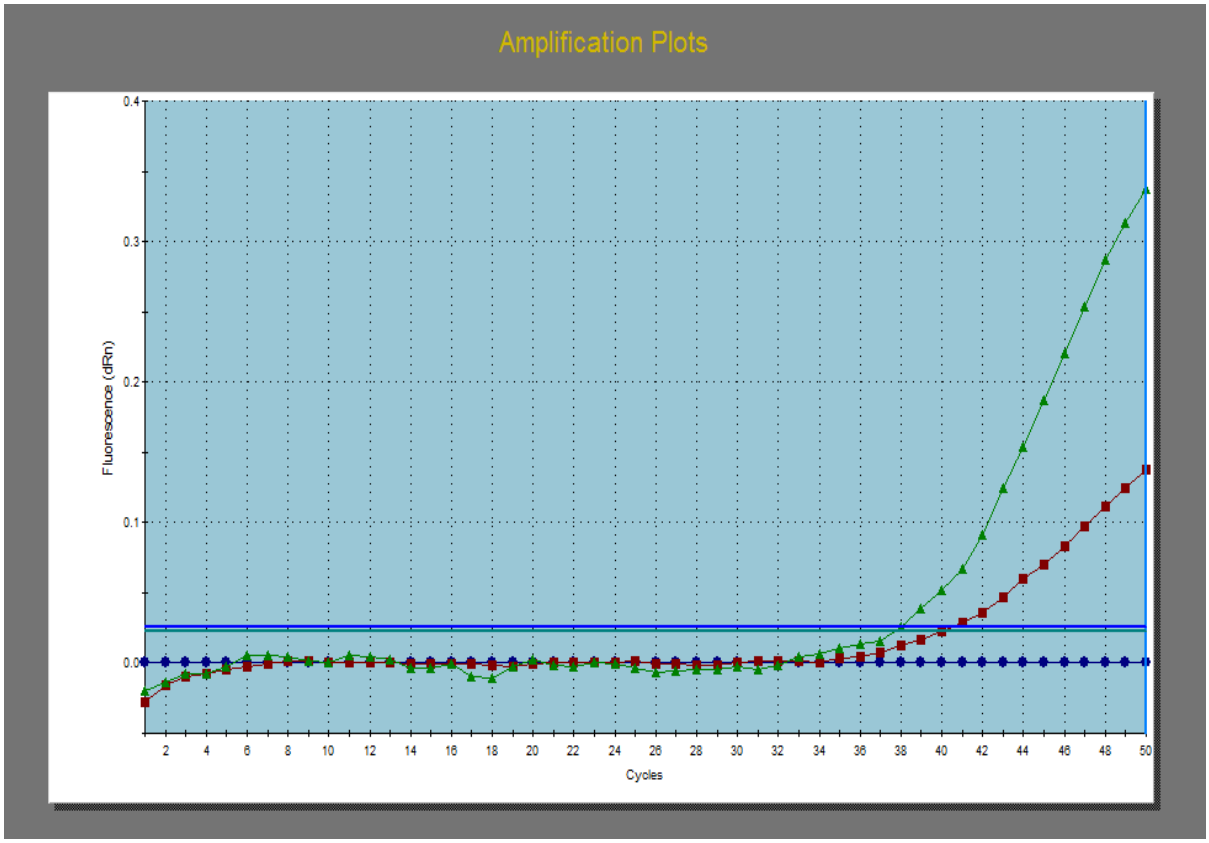


**Figure 8.**

**Real-time PCR result for *CY2C19*17*17***

(homozygous for variant **17*)

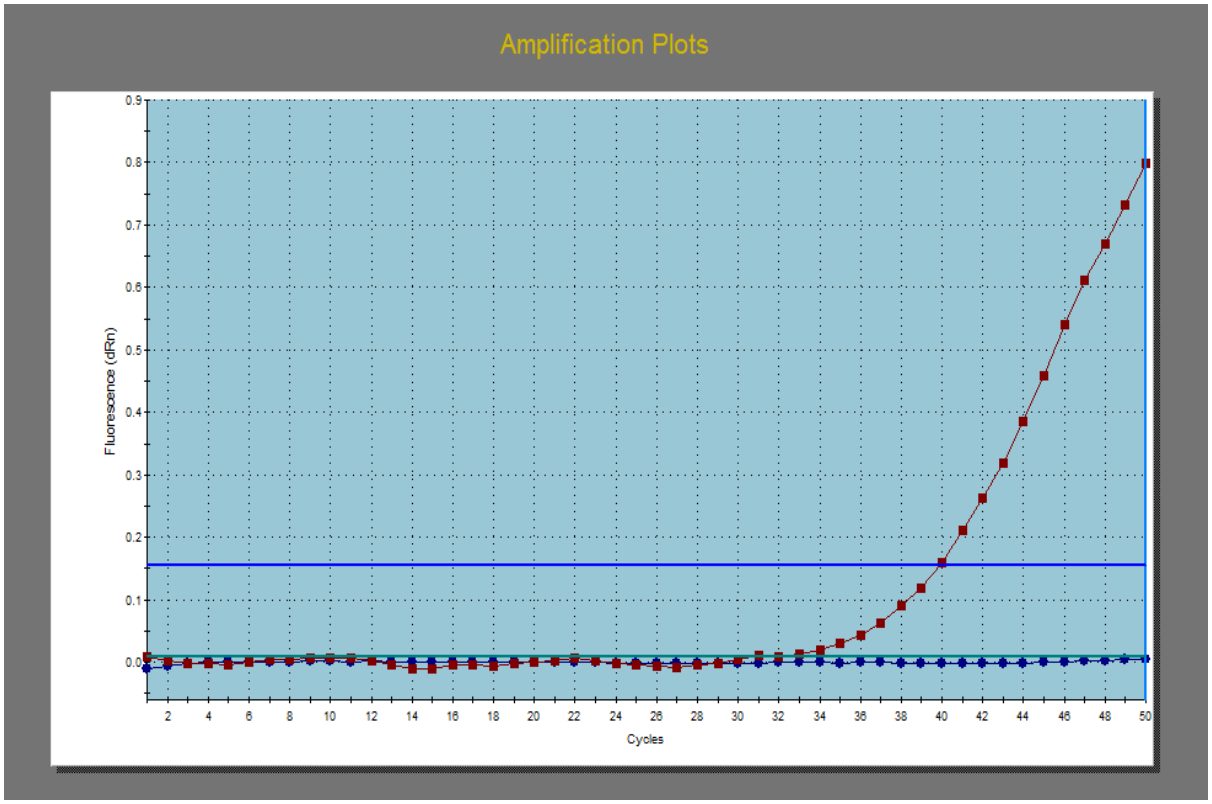


**Figure 9.**

### 4.4.1.3 *CYP3A5*3* genotyping

Multiplication of the *CYP3A5*3* variant, was performed using 10.5 µl PCR mix [3.625 µl $H_2O$, 6.25 µl master-mix (DNA polymerase, dATP, dTTP, dGTP, dCTP) and 0.625 µl of gene specific primer] together with 2 µl of participant's DNA and thereafter undergoing incubation according to specific conditions.

Real Time PCR thermal conditions for *CYP3A5*3* variant were:

2 minutes (50°C)

10 minutes (95°C),

15sec (92°C) } 50 cycles

60sec (60°C) }

**Table 11. *CYP3A5* 3* variant**

**SNP ID:** rs776746
**Assay code:** C_26201809_30
**Gene:** *CYP3A5*
**Gene Name:** cytochrome P450, family 3, subfamily A, polypeptide 5
**Set Membership**: > HapMap > DME > Validated > Inventoried
**Chromosome Location**: Chr.7: 99270539 - 99270539 on NCBI Build 37
**Polymorphism:** T/C, Transition Substitution
**Context sequence [VIC/FAM] is T**/C
ATGTGGTCCAAACAGGGAAGAGATA**[T/C]**TGAAAGACAAAAGAGCTCTTTAAAG
***T*-allele frequency in Caucasians is 0.01 (or 1%)**

***C/C*-**Homozygous for variant allele
***T/C*-**Heterozygous
***T/T*-**Homozygous for normal allele

**Total** number of samples = 234
***C/C*=230 Samples (460 variant alleles)**
***T/C*=0 Samples (0 variant alleles)**
***T/T*= 4 Samples**

***C* allele frequency in this study=460/468=98.3%**
***T* =8/468=1,7%**

**(**https://www.thermofisher.com/order/genome-database/browse/snp/keyword/C_26201809_30)

**Real-time PCR result for *CY3A5*3*3***

(homozygous for variant **3*)

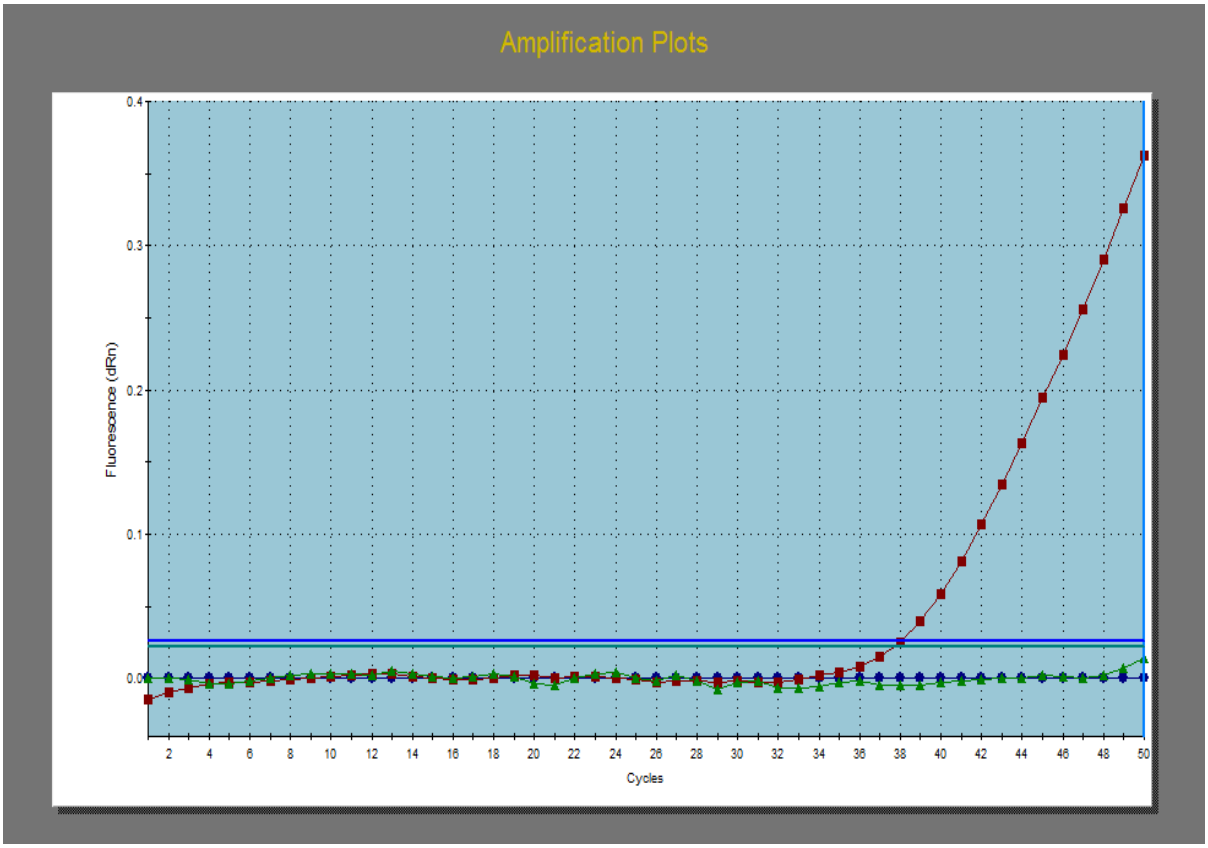


**Figure 10.**

**Real-time PCR result for *CY3A5*1*1***

(homozygous for normal allele)

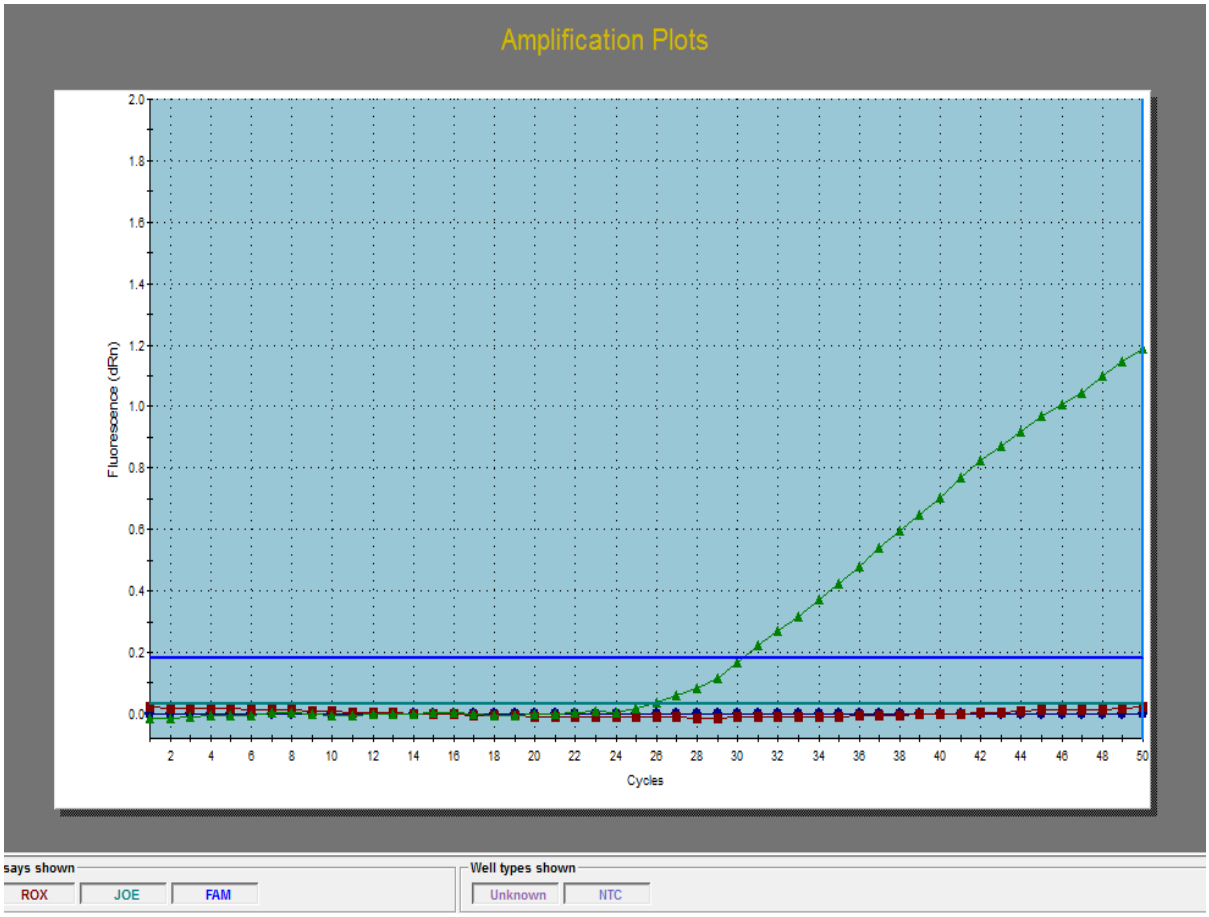


**Figure 11.**

### 4.4.1.4 *VKORC1* (1173C>T) genotyping

In order to multiply *VKORC1 (C>T),* 10,5 µl PCR mix [3.625 µl $H_2O$, 6.25 µl master-mix (DNA polymerase, dATP, dTTP, dGTP, dCTP) and 0.625 µl of gene specific primer] together with 2 µl of participant's DNA, underwent incubation according to specific conditions.

Real Time PCR thermal conditions for VKORC1 (C>T) were:

2 minutes (50°C)

10 minutes (95°C),

15sec (92°C) } 50 cycles

60sec (60°C) }

**Table 12. *VKORC1* (1173C>T or C6484T)**

**SNP ID:** rs9934438
**Assay code:** C_30204875_10
**Gene:** *VKORC1*
**Gene Name:** protease, serine, 53
**Set Membership**: > HapMap > DME > Validated > Inventoried
**Chromosome Location**: Chr.16: 31104878 - 31104878 on NCBI Build 37
**Polymorphism:** A/G, Transition Substitution
**Context sequence [VIC/FAM] is A**/G
CCCCGACCTCCCATCCTAGTCCAAG**[A/G]**GTCGATGATCTCCTGGCACCGGGCA
***A*-allele frequency in Caucasians is 0.38 (or 38%)**

***G/G*-**Homozygous for normal allele
***G/A*-**Heterozygous
***A/A*-**Homozygous for variant allele

**Total** number of samples = 234
***G/G*=78 Samples**
***G/A*=121 Samples (121 variant alleles)**
***A/A*= 35 Samples (70 variant alleles)**

***A*** allele in this study**=191/468=40,81%**
**(**https://www.thermofisher.com/order/genome-database/browse/snp/keyword/C_30204875_10)

**Real-time PCR result for *VKORC1 (A/A)***

(homozygous for variant allele)

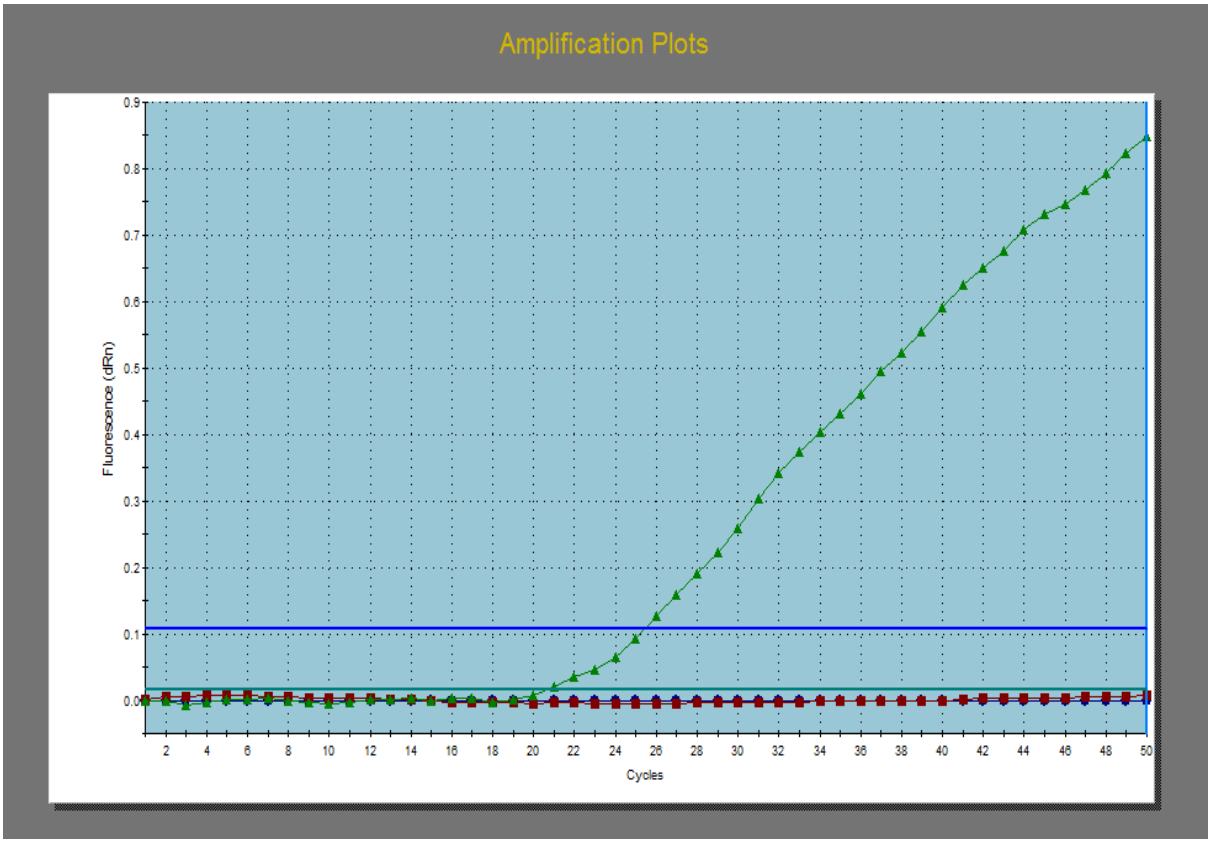


**Figure 12.**

**Real-time PCR result for *VKORC1 (G/A)***

(heterozygous for variant allele)

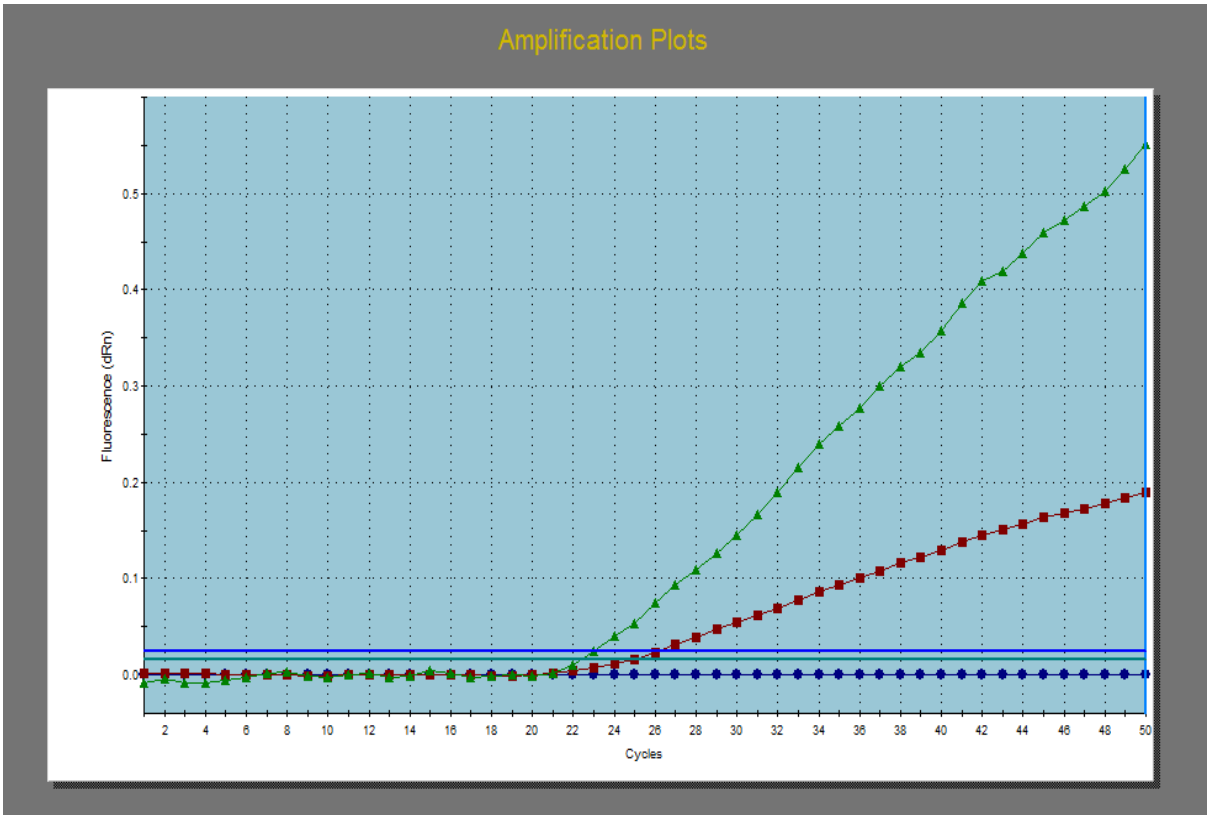


**Figure 13.**

**Real-time PCR result for *VKORC1 (G/G)***

(homozygous for normal allele)

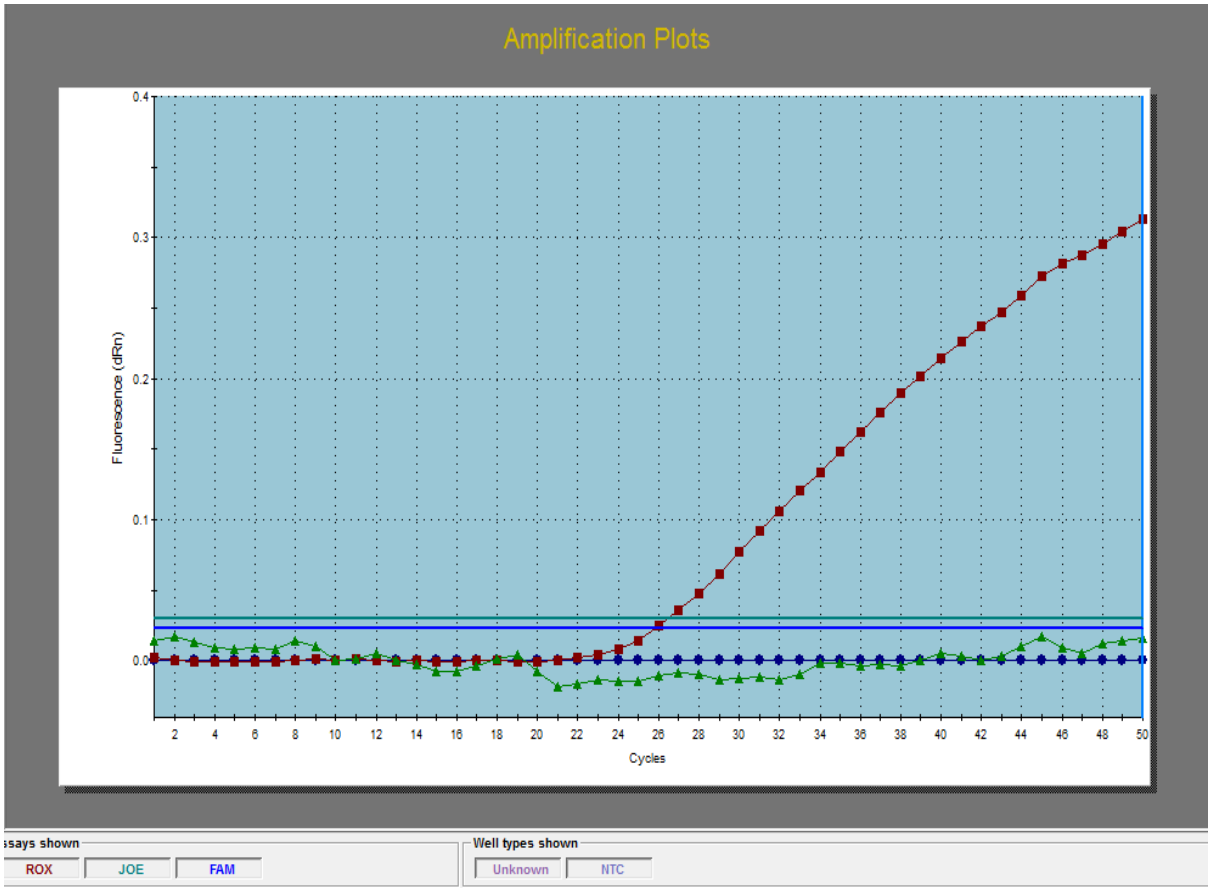


**Figure 14.**

## 4.5 STATISTICAL ANALYSIS

This study's statistics were conducted through a descriptive analysis of all the analyzed variables. The gene-counting method was used to estimate the allele frequency. Likewise, this study compared the observed allele and genotype frequency and expected allele and genotype frequencies according to the Hardy-Weinberg equilibrium. The frequency of specific genotypes and haplotypes in healthy Kosovo population and other populations was compared using the proportion test. Chi-squared test and proportion test were used in analysing frequency and distribution of genotypes as well as phenotyping groups such as extensive, intermediate, ultra-extensive and poor metabolizers. In addition, a 95% confidence interval was assigned.

Interpretation of results is set at a 5% significance level ($p<0,05$). The SPSS statistical (SPSS Inc., Chicago, IL, USA, 20.0 version) program and the online calculator (Link 5) were used for conducting statistical data processing.

# 5. RESULTS

**Table 13.**

| **Demographic data regarding the study group' participants** | |
|---|---|
| Total number of participants | 234 |
| Sex (male/female) | 118/116 |
| Age (years) (mean ± SD) | 36.01 ± 12.70 |
| Age range (years) | 20 - 65 |

## 5.1 Frequency of *CYP2C9, CYP2C19, CYP3A5* and *VKORC1* polymorphism in Kosovo's healthy population

Genotyping for *CYP2C9, CYP2C19, CYP3A5* and *VKORC1* was conducted in 234 healthy Kosovo's healthy volunteers. Statistical analysis of the results was performed in order to find out the eventual difference between Kosovars and other populations regarding their genotypes and haplotypes. In addition, it was investigated whether there is any difference in genotypes and haplotypes between male and female participants of this study.

**Table 14. CYP2C9 genotype, allele and predicted phenotype frequencies in Kosovo's population**

| Gene | Genotype | Number of subjects (n) | Observed frequency (%) | 95% Confidence Interval | Predicted phenotype | Investigated Allele | Number (2n) / (%) |
|---|---|---|---|---|---|---|---|
| ***CYP2C9*** | *CYP2C9*1*1* | 117 | 50,00 | 43.59-56.41 | EM | *CYP2C9*1* | 335 (71.58) |
| | *CYP2C9*1*2* | 62 | 26,49 | 20.84-32.14 | IM | *CYP2C9*2* | 82 (17,52) |
| | *CYP2C9*1*3* | 39 | 16,66 | 11.89-21.43 | IM | *CYP2C9*3* | 51 (10,89) |
| | *CYP2C9*2*2* | 8 | 3.41 | 1.08-5.74 | PM | | |
| | *CYP2C9*2*3* | 4 | 1,70 | 0.04-3.36 | PM | | |
| | *CYP2C9*3*3* | 4 | 1,70 | 0.04-3.36 | PM | | |
| **Total** | | *234 subjects* | | | | | |

**Table 15. CYP2C9 genotype frequencies in Kosovo's population compared to other countries'**

| CYP2C9 genotype | This study (%) | Croatia | Macedonia (%) | Greece (%) | Slovenia (%) | UK (%) | Belgium (%) | Russia (%) | Sweden (%) | Argentina (%) | Africa (%) | Japan (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *CYP2C9*1*1* | 50,00 | 59.72 | 57.4 | 62 | 66.6 | 70.0 | 67 | 68 | 66.7 | 56 | 93.6 | 95 |
| *CYP2C9*1*2* | 26.49 | 23.52 | 21.6 | 20 | 19.4 | 14.2 | 18,2 | 18.2 | 18.6 | 23 | 4.2 | 0 |
| *CYP2C9*1*3* | 16,66 | 12.78 | 11.3 | 13.5 | 10.8 | 14.2 | 11,3 | 11.3 | 11.6 | 5 | 2.1 | 4 |
| *CYP2C9*2*2* | 3.41 | 1.76 | 2.84 | 1.5 | 1.5 | 0.8 | 0 | 0.6 | 0.4 | 14 | 0 | 0 |
| *CYP2C9*2*3* | 1.70 | 1.94 | 1.13 | 2.8 | 1.5 | 0.8 | 1,6 | 1.2 | 1.6 | 1 | 0 | 0 |
| *CYP2C9*3*3* | 1.70 | 0.28 | 1.13 | 0 | 0 | 0 | 0,8 | 0.3 | 0.6 | 0 | 0 | 1 |
| ***Total*** | 234 | 1080 | 179 | 283 | 129 | 120 | 121 | 290 | 430 | 101 | 47 | 828 |
| ***Reference*** | - | [a] | [j] | [b] | [c] | [d] | [e] | [f] | [f] | [g] | [h] | [i] |

a-Ganoci L, Bozina N et al 2017; b- Arvanitidis K et al 2007; c- Herman D et al 2003; d- Biss TT et al 2011; e- Buzoianu, A. D. et al 2012; f- Azarpira N et al 2010; g- Scibona P et al 2012; h- Isaza C et al 2007; i- Mushiroda T, et al 2006; j- Jakovski K et al 2013;

**Table 16. CYP2C9 allele frequencies in Kosovo's population compared to other countries'**

| **CYP2C9 allele** | **This study** (%) | **Croatia** | **Slovenia** (%) | **Macedonia** (%) | **Greece** (%) | **France** (%) | **Romania** (%) | **China** (%) | **Japan** (%) | **Benin (Africa)** (%) | **Iran** (%) | **Argentina** (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *1 | 71.58 | 79 | 81,7 | 78.8 | 79 | 77 | 79.4 | 96.3 | 97,6 | 95,5 | 64,8 | 70,79 |
| *2 | 17,52 | 14 | 12 | 13.9 | 12.8 | 15 | 11.3 | 0.1 | 0 | 0 | 25,3 | 25,74 |
| *3 | 10,89 | 7 | 6,2 | 7.3 | 8,1 | 8 | 9.3 | 3.6 | 2,3 | 0 | 9,8 | 2,97 |
| **Total** | 234 | 200 | 129 | 179 | 283 | 151 | 332 | 394 | 828 | 111 | 150 | 101 |
| ***Reference*** | - | [a] | [c] | [d] | [b] | [h] | [e] | [j] | [i] | [e] | [f] | [g] |

a-Ganoci L, Bozina N et al 2017; b- Arvanitidis K et al 2007; c- Herman D et al 2003; d- Jakovski K et al 2013; e- Buzoianu, A. D. et al 2012; f- Azarpira N et al 2010; g- Scibona P et al 2012; h-Yang JQ et al 2003; i- Mushiroda T, et al 2006;

**Table 17. Genotype, allele and predicted phenotype frequencies of CYP2C19 in the Kosovo population**

| Gene | Genotype | Number of subjects (n) | Observed frequency (%) | 95% Confidence Interval | Predicted phenotype | Investigated allele | Number (2n) / (%) |
|---|---|---|---|---|---|---|---|
| **CYP2C19** | *CYP2C19*1*1* | 107 | 45,72 | 39.34-52.1 | EM | *CYP2C19*1* | 318 (67.94) |
| | *CYP2C19*1*2* | 43 | 18,37 | 13.41-23.33 | IM | *CYP2C19*2* | 61 (13,03) |
| | *CYP2C19*2*2* | 5 | 2,13 | 0.28-3.98 | PM | *CYP2C19*17* | 89 (19,01) |
| | *CYP2C19*2*17* | 8 | 3,41 | 1.08-5.74 | IM | | |
| | *CYP2C19*1*17* | 61 | 26,06 | 20.44-31.68 | UM | | |
| | *CYP2C19*17*17* | 10 | 4,27 | 1.68-6.86 | UM | | |
| **Total** | | ***234 subjects*** | | | | | |

**Table 18. CYP2C19 genotype frequencies in Kosovo's population compared to other countries'**

| CYP2C19 genotype | This study (%) | Greece (%) | Macedonia (%) | Croatia | Russia (%) | Slovenia (%) | Iran (%) | Mexico % | Italy (%) | Colombia (%) | India (%) | China (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1*1* | 45.72 | 44.17 | 41.8 | 36.03 | 32.65 | 68.2 | 41.7 | 60.08 | 79.4 | 83.5 | 16.1 | 42 |
| **1*2* | 18.37 | 17.8 | 19.0 | 19.56 | 16.99 | 30 | 18.3 | 13.03 | 18.8 | 15.3 | 31.0 | 41 |
| **2*2* | 2.13 | 2.1 | 2.7 | 2.4 | 1.44 | 0.7 | 2.2 | 0.42 | 0 | 1 | 18.4 | 3 |
| **1*17* | 26.06 | 28.6 | 28.3 | 31.34 | 32.95 | / | 28.8 | 21.01 | / | / | 20.7 | 4 |
| **2*17* | 3.41 | 4.3 | 4.3 | 5.29 | 8.03 | / | 3.3 | 3.36 | / | / | 12.6 | 2 |
| **17*17* | 4.27 | 3.2 | 3.8 | 5.39 | 6.79 | / | 5.5 | 2.1 | / | / | 1.2 | / |
| **Total** | *234* | *283* | *184* | *1002* | *971* | *129* | *180* | *238* | *360* | *189* | *20* | *100* |
| Reference | - | (a) | (b) | (k) | (c) | (e) | (d) | (f) | (g) | (h) | (i) | (j) |

a-Ragia G et al 2009; b- Jakovski K et al 2013; c- Sychev DA et al 2015; d- Payan M et al 2015; e- Herman D et al 2003; f- Favela-Mendoza AF et al 2015; g- Scordo MG et al 2004; h- Isaza C et al 2007; i- Anichavezhi D et al 2012; j- Zhou Q et al 2009; k-Ganoci L, Bozina N et al, 2017

**Table 19. CYP2C19 allele frequencies in Kosovo's population compared to other countries'**

| CYP2C19 allele | This study (%) | Greece (%) | Sweden (%) | Croatia (%) | Macedonia (%) | Faroe Island (%) | Mexico (%) | China (%) | Tibet (%) | Iran (%) | Africa (%) | Japan (%) | India (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1* | 68.16 | 67.32 | 64 | 61.3 | 65.4 | 65.9 | 77.10 | 67.50 | 78.13 | 65.3 | 63.0 | 58 | 42.0 |
| **2* | 13.03 | 13.07 | 16 | 15 | 14.4 | 18.7 | 8.61 | 25.5 | 15.1 | 13.1 | 12.0 | 27.9 | 40.2 |
| **17* | 19.01 | 19.61 | 20.0 | 23.7 | 20.2 | 15.4 | 14.29 | 3.0 | 1.56 | 21.6 | 19.0 | 1.3 | 17.9 |
| **Total** | *234* | *283* | *185* | *1002* | *184* | *312* | *238* | *100* | *96* | *180* | *149* | *265* | *216* |
| Reference | - | (a) | (k) | (e) | (b) | (l, m) | (f) | (j) | (g) | (h) | (c) | (d) | (i) |

a-Ragia G et al 2009; b- Jakovski K et al 2013; c- Strom CM et al 2012; d- Sugimoto K et al 2008; e-Ganoci L, Bozina N et al, 2017; f-Favela-Mendoza AF et al 2015; g- Jin T et al 2016; h-Payan M et al 2015; i- Anichavezhi D et al 2012; j- Zhou Q et al 2009; k- Ramsjö M et al 2010; l-Halling J et al 2005; m- Kurose K et al 2012;

**Table 20. CYP3A5 genotype frequency in Kosovo's population**

| Genotype | Number of subjects (n) | Observed frequency (%) | 95% Confidence Interval | Predicted phenotype | Investigated allele | Number (2n) / (%) |
|---|---|---|---|---|---|---|
| **1/*1* | 4 | 1.7 | 0.04-3.36 | expressor | *CYP3A5*1* | 8 (1.7) |
| **1/*3* | 0 | 0 | 0 | expressor | *CYP3A5*3* | 460 (98.3) |
| **3/*3* | 230 | 98.3 | 96.64-99.96 | non-expressor | | |
| *Total* | 234 | | | | | |

**Table 21. CYP3A5*3 variant frequency (6986A>G) in Kosovo and other countries population**

| CYP3A5 | Present study | Macedonia | Poland | Greece | Tanzania/ (Afro-Americans) | China | India | Sweden |
|---|---|---|---|---|---|---|---|---|
| *1** | 1.7% | 1.1% | 6% | 5.7 % | 51.0% | 24.5% | 40.6% | 7 % |
| *3** | 98.3 % | 90.8 % | 94 % | 94,3 % | 19.0 % | 75.5 % | 59.4% | 93 % |
| n | **234** | 194 | 200 | 283 | 144 | 108 | 90 | 136 |
| *Reference* | - | [a] | [b] | [c] | [d] | [e] | [e] | [d] |

a-Jakovski K et al 2012; b- Adler G et al 2009; c- Arvanitidis K et al 2007; d- Mirghani RA et al 2006; e- Balram C et al 2003.

**Table 22. *VKORC1* genotype and variant allele frequency in Kosovo's population**

| Genotype | Number/ (%) | Allele | 2n / % |
|---|---|---|---|
| *TT (or AA)* | 35 (14.95) | *C* | 277 (59.19) |
| *CT (or AG)* | 121 (51.70) | *T* | 191 (40,81) |
| *CC (or GG)* | 78 (33,33) | | |
| *Total* | 234 | | |

**Table 23. *VKORC1* genotype and allele frequencies in Kosovo compared to other countries' population**

| SNP | Genotype / allele | Ethnic group | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **Present study** | **Croats** (a) | **Italians** (b) | **Chinese** (c) | **Australians** (d) | **Japanese** (e) | **African Americans** (f) |
| | | n / (%) | n / (%) | n / (%) | n / (%) | n / (%) | n / (%) | n / (%) |
| *VKORC1 1173C >T* | *CC* | **78** (33.33) | **63** (33.9%) | **114** (43.2) | **0** (0.00) | **6** (35.29) | **2** (1.25) | **181** (80.4) |
| | *CT* | **121** (51.70) | **87** (46.8%) | **116** (43.9) | **47** (16.91) | **6** (35.29) | **22** (13.75) | **42** (18.7) |
| | *TT* | **35** (14.95) | **36** (19.4%) | **34** (12.9) | **231** (83.09) | **5** (29.41) | **136** (85) | **2** (0.9) |
| | *C* (2n) | 277 (59.19) | 213 (57.25%) | 344 (65.15) | 47 (8.46) | 18 (52.94) | 26 (8.13) | 404 (89.78) |
| | *T* (2n) | 191 (40,81) | 159 (42.74) | 184 (34.85) | 509 (91.54) | 16 (47.05) | 294 (91.87) | 46 (10.22) |
| | **Total (n)** | **234** | **186** | **264** | **278** | **17** | **160** | **225** |

a- Mandic D et al, 2015; b- Mazzaccara C et al 2013; c-Yan X et al, 2015; d- Madison J et al 2012; e- Miyagata Y et al, 2011; f- Limdi NA et al, 2008;

**Table 24. Comparison of *CYP2C9, CYP2C19, CYP3A5 and VKORC1* genotypes and alleles between healthy male and female in Kosovo's population**

**(**SPSS Inc., Chicago, IL, USA, 20.0 version**;** https://www.allto.co.uk/tools/statistic-calculators/confidence-interval-for-proportions-calculator/)

| **Gene and genotypes** (this study) | **Male** | | | **Female** | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | n | % | 95% CI | n | % | 95% CI | $\chi^2$ | p value | Reference |
| **CYP2C9 genotypes** | | | | | | | | | |
| **1*1* | 57 | 48.3 | 39.28 - 57.32 | 60 | 51.7 | 42.61- 60.79 | 0.25 | 0.62 | Present study |
| **1*2* | 34 | 28.8 | 20.63 - 36.97 | 28 | 24.1 | 16.32-31.88 | 0.64 | 0.42 | |
| **1*3* | 21 | 17.8 | 10.9 - 24.7 | 18 | 15.5 | 8.91-22.09 | 0.25 | 0.62 | |
| **2*2* | 4 | 3.4 | 0.13 - 6.67 | 4 | 3.44 | 0.12 - 6.76 | 0.0 | 1.0 | |
| **2*3* | 1 | 0.84 | -0.81 - 2.49 | 3 | 2.6 | -0.3 - 5.5 | 1.0 | 0.32 | |
| **3*3* | 1 | 0.84 | -0.81 - 2.49 | 3 | 2.6 | -0.3 - 5.5 | 1.0 | 0.32 | |
| **Total** | *n=118* | | | *n=116* | | | | | |
| **CYP2C9 alleles (n)** | | | | | | | | | |
| **1* | 169 | 71.6 | 65.85-77.35 | 166 | 71.55 | 65.74-77.36 | 0.0 | 1.0 | Present study |
| **2* | 43 | 18.2 | 13.28-23.12 | 39 | 16.8 | 11.99-21.61 | 0.16 | 0.69 | |
| **3* | 24 | 10.17 | 6.31%-14.03 | 27 | 11.6 | 7.48 - 15.72 | 0.25 | 0.62 | |
| **Total** | *2n=236* | | | *2n=232* | | | | | |
| **CYP2C19 genotypes** | | | | | | | | | |
| **1*1* | 51 | 43.2 | 34.26-52.14 | 56 | 48.3 | 39.21 - 57.39 | 0.64 | 0.42 | Present study |
| **1*2* | 24 | 20.3 | 13.04-27.56 | 19 | 16.4 | 9.66 - 23.14 | 0.64 | 0.42 | |
| **2*2* | 1 | 0.84 | -0.81 - 2.49 | 4 | 3.44 | 0.12 - 6.76 | 1.96 | 0.16 | |
| **2*17* | 5 | 4.2 | 0.58 - 7.82 | 3 | 2.6 | -0.3 - 5.5 | 0.49 | 0.48 | |
| **1*17* | 31 | 26.3 | 18.36-34.24 | 30 | 25.9 | 17.93 - 33.87 | 0.01 | 0.92 | |
| **17*17* | 6 | 5.1 | 1.13 - 9.07 | 4 | 3.44 | 0.12 - 6.76 | 0.36 | 0.55 | |
| **Total** | *n=118* | | | *n=116* | | | | | |
| **CYP2C19 alleles (n)** | | | | | | | | | |
| **1* | 157 | 66.52 | 60.5 - 72.54 | 161 | 69.39 | 63.46 - 75.32 | 0.49 | 0.48 | Present study |
| **2* | 31 | 13.13 | 8.82 - 17.44 | 30 | 12.93 | 8.61 - 17.25 | 0.01 | 0.92 | |
| **17* | 48 | 20.33 | 15.2 - 25.46 | 41 | 17.67 | 12.76 - 22.58 | 0.49 | 0.48 | |
| **Total** | *2n=236* | | | *2n=232* | | | | | |
| **CYP3A5*3** | | | | | | | | | |
| *C/C* | 117 | 99.15 | 97.49-100.81 | 113 | 97.4 | 94.5-100.3 | 1.0 | 0.32 | Present study |
| *C/T* | / | / | / | / | / | / | / | / | |
| *T/T* | 1 | 0.85 | -0.81 - 2.51 | 3 | 2.6 | -0.3 - 5.5 | 1.0 | 0.32 | |
| **Total** | *n=118* | | | *n=116* | | | | | |
| *C* | 234 | 99.15 | 97.98- 100.32 | 226 | 97.4 | 95.35 - 99.45 | 2.25 | 0.13 | |
| *T* | 2 | 0.85 | -0.32 - 2.02 | 6 | 2.6 | 0.55 - 4.65 | 2.25 | 0.13 | |
| **Total** | *2n=236* | | | *2n=232* | | | | | |
| **VKORC1** | | | | | | | | | |
| *C/C* | 36 | 30.5 | 22.19 - 38.81 | 42 | 36.2 | 27.45- 44.95 | 0.81 | 0.37 | Present study |
| *C/T* | 63 | 53.4 | 44.4 - 62.4 | 58 | 50.0 | 40.9 - 59.1 | 0.25 | 0.62 | |
| *T/T* | 19 | 16.1 | 9.47 - 22.73 | 16 | 13.8 | 7.52 - 20.08 | 0.25 | 0.62 | |
| **Total** | *n=118* | | | *n=116* | | | | | |
| *C* | 135 | 57.2 | 50.89 - 63.51 | 142 | 61.2 | 54.93- 67.47 | 0.81 | 0.37 | |
| *T* | 101 | 42.8 | 36.49 - 49.11 | 90 | 38.8 | 32.53- 45.07 | 0.81 | 0.37 | |
| **Total** | *2n=236* | | | *2n=232* | | | | | |

### 5.1.1. CYP2C9

This study investigated the *CYP2C9 *2* and **3* variant allele. The frequencies of CYP2C9 alleles were as follows: *CYP2C9*2*=17.52% (mean frequency in Caucasians is 17%) and *CYP2C9*3*=10.89% (mean frequency in Caucasians is 10%). *CYP2C9*1* is present with 71.58%. 117 subjects (50.00%) were EM (*CYP2C9*1/*1*), 101 (43.15%) with the *CYP2C9*1/*2* or *CYP2C9*1/*3* genotype were classified as IM, while 16 subjects (6.81%) with the *CYP2C9*2/*2*, *CYP2C9*2/*3* or *CYP2C9*3/*3* genotype were PM. Result details are shown in tables 14-16. Statistical analysis didn't show any significant difference between male and female participants concerning allele or genotype frequency (Table 23).

### 5.1.2. CYP2C19

The *CYP2C19 *2* and *CYP2C19 *17* allele were the main target of this investigation. The **3* variant allele of *CYP2C19* was not investiagted because it was not found or was extremely rare in Caucasian populations. *CYP2C19*1* allele frequency in healthy Kosovars was 68.16%. **2* and **17* allele frequency was 13.03% and 19.01% respectively. EM that are carriers of two normal alleles (*CYP2C19*1/*1*) in the present study participated with 45.72% (107 subjects), while heterozygous participants (*CYP2C19*1/*2* and *CYP2C19*2/*17*) that were classified as IM participated with 21.78% (51 subjects). 5 subjects (2.13%) with the *CYP2C19*2/*2* genotype were PM. 71 subjects (30.33%) with the *CYP2C19*1/*17* or *CYP2C19*17/*17* genotype were classified as UM, among them 10 subjects (4.27%) were homozygous carriers of **17* allele. 8 subjects (3.41%) were carriers of the combined genotype *CYP2C19*2/*17* (Table 17 and 18).
For *CYP2C19* alleles and genotypes was found no significant difference between male and female participants of present study (Table 23).

### 5.1.3. CYP3A5

Considering its importance, we investigated the frequency of the *CYP3A5*3* allele. The frequency of genotypes of *CYP3A5*3* allele in this research were as follows: 230 subjects (98%) carried the *CYP3A5*3/*3* genotype and were classified as non-expressors, 4 subjects (2%) were carriers of the *CYP3A5*1/*1* genotype and classified as expressors, while the *CYP3A5*1/*3* genotype was not found (Table 19 and 20). *CYP3A5*3*'s frequency was 98.3%. We found no significant difference between participants of both sexes (Table 23).

### 5.1.4. VKORC1

The focus of this investigation was the *1173C>T* (or C6484T) variant of *VKORC1*. Given that the *VKORC1 1173C>T* variant is in linkage disequilibrium with the *-1639 G > A* (or G3673A) variant, we decided to investigate *VKORC1 1173C>T* alone. 78 (33,3%) subjects were homozygous for 1173*C*, 121 (51,7%) were heterozygous (*CT)* and 35 (14.95%) were homozygous for 1173*T* (Table 21 and 22). In our study, there was no significant difference between subjects of both sexes (male vs female) with regard to genotypes and alleles frequency (Table 23).

# 6. DISCUSSION

The concept of "personalized medicine has been active in medical practice" in recent years. A better understanding of interindividual differences in DNA sequence has improved our ability to associate the effect of a medicinal product on certain gene variants, which is the subject of pharmacogenetics/pharmacogenomics. This has resulted in a greater emphasis of the pharmaceutical industry, academia, healthcare professionals and regulators on thestudy of the genetic basis of variability in response to pharmacotherapy. While scientific knowledge in this area is well documented, knowledge transfer and application in clinical practice is slow. This has resulted in the establishment of a network of societies the main objective of which is to issue recommendations and clear guidelines on how to adjust therapy according to the results of pharmacogenetic analysis. One of the most prominent ones is the Pharmacogenomics Knowledge Base association -PharmGKB (http://www.pharmgkb.org). On its website, users can find the latest information regarding molecular mechanisms of different pharmacogenes, as well as their inputs in pharmacotherapy. The database allows searches of genes and their variants, biochemical pathways as well as medicinal products and diseases. A consortium entitled Pharmacogenetics Clinical Implementation Consortium (CIPC) has been established with the purpose to collect and implement knowledge. The main objective of the CPIC is to issue peer-reviewed, updated, evidence-based and freely available instructions and guidelines for the use of medicines according to the results of genetic tests. Further progress in the individualization of therapy is directed towards the development of medicines appropriate for specific subpopulations of patients. There are a number of examples showing the benefits of an individualized approach to choosing a medicine and its dose compared to the approach based on the results of population-level pharmacokinetic studies. Pharmacogenomics represents an important link in "personalized medicine" with emphasis on genomic and epigenomic factors that influence pharmacokinetics and pharmacodynamics and is also important in understanding drug interactions. While our knowledge of epigenomics as a modulator of pharmacotherapy efficiency is still in the developmental stage, a large amount of information about the impact of genomic factors is readily available. It is believed that since genetic factors account for about 25% - 50% of all the unexpected reactions to a medicinal product, it may negatively affect the efficacy of a medicinal

product and increase the risk of adverse reactions. Integrating genomic biomarkers in clinical and other studies, as well as information on methodologies used, must follow certain principles that take into account and consider the impact of biomarkers in the study and analysis of outcomes with the intention of maximizing the benefits for the patient. Important emphasis in association studies is placed on phenotypic data that need to comply with the international standardized criteria.

Many investigations proved that ethnic specificities have a direct impact on pharmacogenetic characteristics. This study isthe first investigation which explored the distribution and frequency of variant alleles of enzymes that have a crucial role in biotransformation of a large number of xenobiotics (including drugs) in Kosovo's healthy population. The basic purpose was to explore distribution of important biomolecular markers in order to determine the risk group of individuals concerning the drug dosing and, consequently, drug's side effects.

## 6.1. Phase I metabolism interethnic variability

Human beings as other living organisms have developed different metabolic pathways to eliminate products of metabolism (toxins). Many metabolic enzymes characterized by different or partial similarities regarding catalytic properties have a key role in the process of detoxication and elimination of metabolic products. In the past, evolution of those genes that code the CYP 450 superfamily of enzymes has made possible the survival of the human being in different habitats and usage of food that contained hazardous chemicals. It is believed that on earth, the ancestral *CYP 450* gene has been arisen nearly 3,5 billion years ago, and over the time has undergone evolutionary changes that has made possible various functions for which it is known. In the evolutionary process of living species, including human beings, species have survived because they gained the ability to metabolize xenobiotics at the level that made possible their survival. Nevertheless, living species, including human beings, differ significantly (Guengerich and al 1987; Danielson PB, 2002). Recently, it is these exact evolutionary processes (Hiratsuka M, 2016) that explain the documented interethnic differences in drug metabolism in humans.

### 6.1.1 Frequency and distribution of genotypes of CYP 450 enzymes and drug target (VKORC1) in Kosovo's healthy population

To date, the majority of European countries have reported the frequency of genetic polymorphisms of the above-mentioned enzymes and drug target. However, this data is missing in the case of Kosovo's population.

In this investigation, for the first time, we determined the frequency of genetic polymorphism of *CYP2C9, CYP2C19, CY3A5* and *VKORC1* in Kosovo's healthy population.

Participants of this study originated from all the parts of Kosovo representing a mixed and appropriate sample of Kosovars. All of them were ethnic Albanians. In most cases, the frequency of variant alleles and genotypes was in accordance with the data from other white Caucasian populations (Buzoianu AD et al 2012).

#### 6.1.1.1 CYP2C9

In Kosovo's population, the frequency of *CYP2C9* variant alleles is in accordance with the mean frequency of these alleles in Caucasians. *CYP2C9*2*=17.52% (mean frequency in Caucasians is 17%) and *CYP2C9*3*=10.89% (mean frequency in Caucasians is 10%) (Applied Biosystems®). Our data correspond to the data on Croats published by Bozina et al (2003) and data on French people published by Yang JQ et al (2003). The frequency of poor CYP2C9 metabolizers in the present study was 6.8%, which is close to that of the Spanish population (5%) (http://www.1000genomes.org/). The prevalence of poor metabolizers (6.8%) in this study is almost similiar to Kosovo's neighbouring countries such as Macedonia (p= 0.3696), Greece (p = 0.2123) or Croatia (p= 0.0857) but on the other side differs significantly compared to Sweden (p = 0.0090), United Kingdom (p=0.0345) or Russia (p= 0.0077) (Arvanitidis K et al 2007; Ramsjö M et al 2010; Korytina G et al 2012; Kapedanovska Nestorovska A et al 2014).

In Caucasians, individuals that possess **2* and **3* variant of *CYP2C9* have a low enzymatic activity compared to subjects that are carriers of two normal alleles. The most common variant allele of the *CYP2C9* gene among Caucasians is *CYP2C9*2*. *CYP2C9*2* and *CYP2C9*3* alleles in Caucasians are present with 12.5-16.5% (*2C9*2*) and 7.1-9.5% (*2C9*3*) respectively (Bozina

et al 2003; Yang JQ et al 2003; Sánchez-Diz P et al 2009). The frequency of *CYP2C9*2* in the Mediterranean European countries and other parts of Europe was: Spain (16%), Greece (12.9%), Croatia (14%), Romania (11.3%), Germany (14.0%), France (15%) and Sweden (10.7%) (Yasar U et al 1999; Burian M et al 2002; Dorado P et al 2003; Arvanitidis K et al 2007; Buzoianu, A. D. Et al 2012; Ganoci, L. et al 2017).

The *CYP2C9*2* variant is rare (prevalence: 0 - 0.1%) in Asian populations, especially in countries of Eastern Asia such as Japan, China and Korea. The prevalence of the *CYP2C9*2* variant decreases in the direction from Europe toward Eastern Asia. In Asia, *CYP2C9*3* manifests the highest prevalence in the population of Southern Asia (11.7%) (Céspedes-Garro C et al 2015) while in the Balkans it is present at the following frequency: Croatia 9.5%, Greece 8.13% and Macedonia 7.3% (Bozina et al 2003; Azarpira, Namazi et al. 2010; Buzoianu AD et al 2012). The *CYP2C9*2* and *CYP2C9*3* variants are present at a lower frequency among Africans (3.3% and 2.3%) compared to Caucasians (Yang ZF et al. 2010; Kudzi W et al. 2016).

#### 6.1.1.1.1. Drug Dosing Guidelines

1. As one of the COX-2 inhibitors, celecoxib is metabolized primarily by CYP2C9. The administration of celecoxib in individuals that are previously known or suspected to be poor metabolizers should be done with caution. For this category of individuals, one should consider dose reduction by 50% or alternative management for juvenile rheumatoid arthritis (On FDA Biomarker List).
2. In vitro studies have shown that CYP2C9 is an important enzyme in the metabolism of flurbiprofen (NSAIDs). Flurbiprofen should be administered with caution in poor CYP2C9 metabolizers because decreased clearance of this drug may generate high plasma levels (On FDA Biomarker List).
3. Drug label for lesinurad (urate transporter inhibitor), which is approved by FDA, states that in CYP2C9 poor metabolizers exposure to the drug is increased. Therefore, in dealing with these individuals, it is necessary to be cautious (On FDA Biomarker List).
4. The FDA-approved drug label for dronabinol (orally active cannabinoid) indicate that individuals who carry variant alleles that result in reduced CYP2C9 function may have two or three fold higher drug level in the blood (On FDA Biomarker List).

5. According to the FDA-approved drug label for piroxicam, CYP2C9 poor and intermediate metabolizers showed higher systemic levels of drug in comparison to normal/extensive metabolizers. Hence, in this category of patients (known or suspected poor CYP2C9 metabolizers) should consider a dose reduction (On FDA Biomarker List).

6. Anticoagulant warfarin routinely is used for prophylaxis and treatment in cases of atrial fibrillation, venous thrombosis, cardiac valve replacement, pulmonary embolism, thromboembolic complications and to reduce reinfarction. Dosing information related to pharmacogenomics for variants of *CYP2C9* and *VKORC1* is provided within the drug label.

The warfarin label states that patients carrying deficient protein C (PROC) or protein S (PROS1) after warfarin administration experienced tissue necrosis (On FDA Biomarker List).

7. The European Public Assessment Report (EPAR) of EMA (European Medicines Agency) for voriconazole lacks pharmacogenetic information. That report contains only warning information concerning co-administration of drugs that are known as substrates, activators or inhibitors of CYP3A4, CYP2C9 or CYP2C19 as a result of drug-drug interactions [voriconazole (VFEND) EMA drug label].

8. Pharmaceuticals and the Medical Devices Agency of Japan (PMDA) notes that patients with the *CYP2C9*1*3* or *CYP2C9*3*3* genotype after receiving celecoxib (brand name: Celecox) had an increased Area Under the Curve (AUC) as compared to individuals containing the *CYP2C9*1*1* genotype (PMDA, Pharmaceuticals and Medical Devices Agency, Japan).

9. Celecoxib (CELEBREX) monograph states that in individuals who are CYP2C9 poor metabolizers, administration of this drug should be done with caution. Additionally, in these individuals just half of the lowest recommended dose of the drug should be administered, with a maximum of 100mg as a daily dose. Nevertheless, the celecoxib monograph does not discuss CYP2C9 genotyping prior to treatment (celecoxib product monograph).

10. The product monograph for anticoagulant warfarin (brand name: COUMADIN) states that individuals that are carriers of two copies of **2* or **3* alleles of CYP2C9 may require reduced mean daily warfarin dose and are exposed to a greater bleeding risk, compared to those that have a *CYP2C9*1*1* genotype. In addition, the presence of *VKORC1* variant alleles such as *-1639G>A,* is associated with adequate changes in the dose requirement of warfarin (Health Canada or Santé Canada).

### 6.1.1.2. CYP2C19

With regard to *CYP2C19*, we found that frequencies of *CYP2C19*2* and *CYP2C19*17* variant were 13.03% and 19.01%, respectively. Study data are comparable to other Caucasians. We found high a similarity of CYP2C19 variant alleles and genotypes between Kosovo, on the one side, and Macedonia (p value for *2C19*2* and *2C19*17* variant was 0.55 and 0.69 respectively) and Greece (p value for **2* and **17* variant of *CYP2C19* was 1.00 and 0.84 respectively), on the other. Our findings also showed a significant difference compared to other Caucasian such as Russians (p value for *CYP2C19*1*1* was 0.0003), or even more difference compared to populations of other continents such as Colombia (South America) or India ($p<0.0001$) (Isaza C et al 2007; Ragia G et al 2009; Anichavezhi D et al 2012; Jakovski K et al 2013; Sychev DA et al 2015).

The *CYP2C19*2* presence in Caucasian populations varies from 13.07-18.7%. The prevalence of *CYP2C19*2* in Asians is 13.1- 42.2% (Anichavezhi D et al 2012; Payan M et al 2015) and even a twofold higher frequency of this allele was observed in Native populations from Oceania (61.30%). In some populations, both the *CYP2C19* variants can have a frequency of up to 90% (Fricke-Galindo I et al 2016). In Africans and African Americans *CYP2C19*2* frequency ranges from 12-15% (Scott SA et al 2011; Strom CM et al 2012). We have found 5 subjects (2,13 %) to be carriers of two **2* allele *(CYP2C19*2/*2* genotype). As both variant alleles are defective (inactivating), it can be rather significant for the administration of antiplatelet drug clopidogrel, which needs to be activated predominantly by CYP2C19 enzyme.

As regards the *CYP2C19*17* variant, its frequency in Caucasians varies from 15.4% to 20.2% (Halling J et al 2005; Jakovski K et al 2013). The frequency of *CYP2C19*17* in Asians and African Americans is 1.3-21% and 19% respectively (Sugimoto K et al 2008; Strom CM et al 2012; Payan M et al 2015). The *CYP2C19*17* allele frequency in Kosovo population was 19.01%, while 10/234 (4.27%) were carriers of *CYP2C19*17/*17* genotype. The frequency of *CYP2C19*17* carriers was 33.74%, with 107 subjects (45,72 %) being homozygous carriers of the *CYP2C19*1/*1* genotype. This data is almost similar to the data on the Croatian, Greek and Macedonian populations. The *CYP2C19*17* allele was documented to be more frequent in

Mediterranean Europeans and inhabitants of Middle East, than in populations of Eastern Asia (Fricke-Galindo I et al 2016).

Even though carriers of the *CYP2C19*1/*17* and *CYP2C19*17/**17 genotype were predicted to be ultrarapid metabolizers, only homozygous carriers of the **17/*17* genotype has been reported to increase the rate of metabolism of CYP2C19 substrates like tricyclic antidepressants (TCAs) and esomeprazole (PPi) compared to extensive metabolizers (EM). Carriers of the *CYP2C19*17* allele who were under the treatment with clopidogrel, were associated with an increased risk of bleeding, especially those who carried the *CYP2C19*17/*17* genotype (Dai ZL et al 2012) who had the highest risk. We have found 8 subjects (3,41 %) who were carriers of the *CYP2C19*2/*17* combined genotype. Metabolic phenotype of individuals that are carriers of **2/*17* genotype is difficult to predict and actually is a matter of debate. Some data suggests that the presence of *CYP2C19*17* allele may not compensate for *CYP2C19*2* (Hicks JK et al 2016).

Because the *CYP2C19*3* variant (c.636G>A; rs4986893) was found extremely rarely in Caucasian populations (0-0.3%) (Gaikovitch EA et al 2003; Scordo MG et al 2004), it was not included in the panel tested in this study. On the contrary, the *CYP2C19*3* variant is very frequent in Asian populations, especially in Japan, Korea and Vietnam (11-14%) (Jin T et al 2016). Approximately, the frequency of the **3* variant among Asians is 2-9% (Scott SA et al 2011), while in Africans this variant is very hard to find (Allabi AC et al 2003).

#### 6.1.1.2.1. Drug Dosing Guidelines

1. Updated CPIC Dosing Guideline for tricyclic antidepressant amitriptyline recommends an alternative drug for poor, rapid or ultrarapid CYP2C19 metabolizers and poor or ultrarapid CYP2D6 metabolizers. For example, in CYP2C19 and CYP2D6, poor metabolizers should consider a 50% dose reduction (Hicks JK et al 2016).

2. Considering that Tricyclic antidepressants (TCAs) have comparable pharmacokinetic properties, it may be reasonable to apply CPIC Dosing Guideline recommendations for amitriptyline and both CYP2C19 and CYP2D6 to other TCAs such as clomipramine, imipramine, doxepine and trimipramine (Hicks JK et al 2016).

3. Another important instruction of CPIC Dosing Guideline is related to clopidogrel. For individuals that are poor or intermediate CYP2C19 metabolizers, this dosing guideline

recommends an alternative antiplatelet drug such as prasugrel or ticagrelor if there is no contraindication (Scott SA et al 2013).

4. The CPIC Dosing Guideline for sertraline (selective serotonin reuptake inhibitor-SSRI), in individuals that are CYP2C19 poor metabolizers recommends selecting an alternative drug which is not metabolized mainly by CYP2C19 enzyme or reduction to 50% of recommended starting dose and titrating to response (Hicks JK et al 2015).

5. For adult patients that are CYP2C19 poor, rapid or ultrarapid metabolizers, the CPIC dosing guideline for triazole antifungal voriconazole recommends choosing an alternative drug whose metabolism is not dependent on CYP2C19 enzyme. A similar treatment strategy should be applied in pediatric poor or ultra-rapid metabolizers. In pediatric patients that are known as rapid metabolizers, the recommended standard dosing should be initiated and thereafter therapeutic drug monitoring (TDM) should be conducted in order to titrate drug's dose to therapeutic trough concentrations (Moriyama B et al 2016).

6. The CPIC Dosing Guideline for the citalopram and escitalopram (SSRI) in CYP2C19 ultrarapid metabolizers, recommends selecting an alternative drug which is not metabolized primarly by CYP2C19. For poor CYP2C19 metabolizers, recommendation is to consider a 50% reduction of standard recommended starting dose and after that to titrate to response or to administer an alternative drug for whose metabolism CYP2C19 is not the major metabolic enzyme (Hicks JK et al 2015).

7. For individuals who are CYP2C19 ultrarapid metabolizers, recommendation is to monitor escitalopram and citalopram plasma concentration and to titrate TC antidepressant's dose up to a maximum of 150% in response to efficacy and adverse drug reactions or to select an alternative drug (Swen JJ et al 2011).

8. With respect to antiplatelet drug clopidogrel, the Dutch Pharmacogenetics Working Group Guideline (DWPG) recommends selection of an alternative drug for poor or intermediate CYP2C19 metabolizers because in these individuals there is an increased risk for reduced response to clopidogrel (Swen JJ et al 2011).

9. Ultrarapid CYP2C19 metabolizers should be informed prior to esomeprazole administration for insufficient response to this drug. Hence, one should consider increasing dose of esomeprazole by 50-100% (Swen JJ et al 2011).

#### 6.1.1.3. CYP3A5

With respect to the *CYP3A5*3* allele, 98.3% of participants in our study had the *CYP3A5*3/*3* genotype and were classified as non-expressors. These data corresponds to the observed prevalence of *CYP3A5*3* in other Caucasians (Kapedanovska Nestorovska A, et al 2014; Ganoci L, et al 2017; Daly AK et al 2015). The prevalence of the *CYP3A5*3* variant in different populations is as follows: in Caucasians its prevalence varies from 85% to 95.5%, in Afro-Americans is 27.9%, in Eastern Asians (Koreans, Chinese and Japanese) is 69-76.7% while in Southern Asians (Indians) is approximately 59.4% (Adler G et al 2009; Ganoci L et al 2017). The present study was conducted in order to explore the frequency of the *CYP3A5*3* allele in Kosovo's population, considering that this variant allele plays an important role in the pharmacokinetics of imatinib (tyrosine kinase inhibitor) which is a drug of choice in pharmacotherapy of blood malignancies such is CML (chronic myeloid leukemia) (Ma LM et al 2015), as well as for tacrolimus, which is a basic therapy for immunosuppression in patients who underwent solid organ and hematopoietic stem cell transplantation. Statistical analysis showed that this variant is more frequent in Kosovars than in other Caucasian populations.

##### 6.1.1.3.1. Drug Dosing Guideline

The CPIC dosing guideline for tacrolimus notes that in individuals who are extensive or intermediate CYP3A5 metabolizers, the starting dose should be 1.5 to 2 fold higher than the recommended starting dose. Beside this, the total starting dose should not exceed 0.3 mg/kg/day. The CPIC dosing guideline for tacrolimus also recommend performing TDM to guide dose adjustment (Birdwell KA et al 2015).

#### 6.1.1.4. VKORC1

*VKORC1* is another gene explored in this study. Exactly, we investigated *1173C>T* (or C6484T) variant in Kosovo's population. 33,3% of subjects were homozygous for 1173*C* allele, 51,7% were heterozygous (*CT*) and 14.95% were homozygous for 1173*T* allele. The *T* allele (variant allele) frequency in our study was 40,81% while for the *C* allele was 59.19%. Genotyping of *VKORC1 1173C>T* (or *-1639 G > A*) and *CYP2C9*3* allele may predict about 50% of the

interindividual variability of the acenocumarol response, providing a much safer anticoagulant therapy (Bodin L et al 2005).

In other populations, frequencies of specific genotypes are as follows: for example, the *TT* genotype of *VKORC1 (1173C>T)* (homozygous for variant allele) in Caucasians appears in approximately of 38% of individuals, whereas in Asians occurs in 91-93% of the population. In Africans, the *TT* frequency is only 0.9% (Limdi NA et al, 2008; Mandic D et al, 2015; Yan X et al, 2015). However, none of the studies has investigated the frequency of major allelic variations of VKORC1 in Kosovo.

Frequencies of *VKORC1* genotypes in this study correspond to the relevant genotypes of the Balkan populations such as Croats (*CC*=33.9%, *CT*=46.8% and *TT*=19.4%), Macedonians (*CC*=39.6%, *CT*=45.5% and *TT*=14.9%) or Romanians (*CC*=32.2%, *CT*=51.2% and *TT*=16.6%) but differ from other European countries such as Italy (*CC*=43.2%, *CT*=43.9% and *TT*=12.9%) (Mazzaccara C et al 2013; Kapedanovska Nestorovska A, et al 2014; Mandic D et al, 2015). Interestingly, comparing our data with that from Australia, we found no significant difference for both genotypes ($p = 0.1164$ for *TT* genotype) and variant alleles ($p = 0.6145$ for *T* allele frequency). On the other hand, comparison of the present study results with the data obtained from China (Asia) and African Americans showed a significant difference ($p<0.0001$) (Yan X et al, 2015; Limdi NA et al, 2008).

#### 6.1.1.4.1. Drug Dosing Guidelines

1. The Clinical Pharmacogenetics Implementation Consortium published an updated guideline for pharmacogenetics-guided warfarin dosing. These recommendations for warfarin dosing are based on the *VKORC1*, *CYP2C9*, *CYP4F2*, and *rs12777823* genotypes and are valid for pediatric and adult patients that share a continental ancestry (Johnson JA et al 2017).

2. Although it has been found that the *VKORC1* genotype is important contributor to the acenocoumarol dose variability, no dosing recommendations are available yet. INR should be checked frequently in those individuals carrying *AA* genotype at rs9934438 (Swen JJ et al 2011).

3. In patients treated with oral anticoagulant phenprocoumon, checking of INR should be performed frequently in those carrying the *AA* genotype at *VKORC1 rs9934438* (Swen JJ et al 2011).

**Table 25. Table presentation of drug labels containing pharmacogenetic (PGx) information approved by FDA (US Food and Drug Administration), EMA (European Medicines Agency), PMDA (Pharmaceuticals and Medical Devices Agency, Japan) and HCSC (Health Canada or Santé Canada)**

| Drug | FDA | EMA | PMDA | HCSC |
|---|---|---|---|---|
| Amitriptyline | Actionable PGx | | | |
| Aripiprazole | Actionable PGx (has dosing information) | Actionable PGx | | Actionable PGx |
| Atorvastatin | Actionable PGx | | Actionable PGx | Actionable PGx |
| Carisoprodol | Actionable PGx | | | |
| Carvedilol | Actionable PGx | | | Actionable PGx |
| Celecoxib | Actionable PGx (has dosing information) | | Actionable PGx | Actionable PGx |
| Citalopram | Actionable PGx (has dosing information) | | | Actionable PGx |
| Clomipramine | Actionable PGx | | | |
| Clopidogrel | Actionable PGx | Actionable PGx | Actionable PGx | Actionable PGx |
| Codeine | Actionable PGx | | Actionable PGx | Actionable PGx |
| Dapsone | Actionable PGx | | Actionable PGx | Actionable PGx |
| Dextromethorphan | Genetic testing recommended | Actionable PGx | | |
| Diazepam | Actionable PGx | | | |
| Erythromycin | Actionable PGx | | | Actionable PGx |
| Ethinyl estradiol | Informative PGx | Genetic testing required | | Actionable PGx |
| Fluoxetine | Informative PGx | | | |

| | | | | |
|---|---|---|---|---|
| Glibenclamide | Actionable PGx | | | Actionable PGx |
| Glimepiride | Actionable PGx | Actionable PGx | | Actionable PGx |
| Glipizide | Actionable PGx | | | |
| Imatinib | Genetic testing required | Genetic testing required | Genetic testing required | Genetic testing required |
| Imipramine | Actionable PGx | | | |
| Indinavir | | Informative PGx | | |
| Irinotecan | Actionable PGx (has dosing information) | | Genetic testing recommended | |
| Lansoprazole | Informative PGx | | | |
| Lidocaine | Actionable PGx | | | |
| Nelfinavir | Informative PGx | Informative PGx | | |
| Omeprazole | Actionable PGx | | Informative PGx | Informative PGx |
| Ondansetron | Informative PGx | | | |
| Pantoprazole | Actionable PGx | | | Genetic testing required |
| Phenytoin | Actionable PGx | | | Genetic testing required |
| Pimozide | Genetic testing required (has dosing information) | | | |
| Propanolol | Informative PGx | Informative PGx | | |
| Quinine | Actionable PGx | | | Actionable PGx |
| Rabeprazole | Actionable PGx | | Actionable PGx | Actionable PGx |
| Ritonavir | | Informative | | |

| | | | | |
|---|---|---|---|---|
| | | PGx | | |
| Sildenafil | | Informative PGx | | |
| Sirolimus | | Informative PGx | | |
| Tamoxifen | Actionable PGx | | | Genetic testing required |
| Telithromycin | | Informative PGx | | |
| Warfarin | Actionable PGx (has dosing information) | | | |
| https://www.pharmgkb.org/view/drug-labels.do | | | | |

### PGx Level

**Genetic testing required**: The label indicate that some kind of tests such as genetic testing, cytogenetic studies, functional protein assays, etc., should be performed before starting to use this drug. This requirement is valid just for a category of patients. PharmGKB considers drug labels that note the variant is an indication for a particular drug, suggesting a required test. If the drug's label notes that a test "should be" conducted, this is also interpreted as a requirement.

**Genetic testing recommended**: The label indicates that some kind of test such as genetic testing, cytogenetic studies, functional protein assays, etc., is recommended before starting the use of this drug. This requirement is valid just for a category of patients. PharmGKB considers drug labels that state the testing "should be considered" to be recommending testing.

**Actionable PGx**: Genetic or other testing related to gene/protein/chromosomal variants are not discussed by the label, but the label does contain information regarding changes in the dose, efficacy or toxicity due to these variants. The label may also state contraindication of the drug in a specific category of patients but does not require or recommend genetic testing, functional protein assays or chromosomal testing.

**Informative PGx**: The label notes either a gene or protein, which has an impact in the metabolism or pharmacodynamics of the drug, but does not contain information suggesting that certain variations related to these genes or proteins leads to variable responses.

# 7. CONCLUSIONS

1. This study provides a useful genetic data regarding the very important drug metabolizing enzymes and drug target. So far, there has not been any information on the pharmacogenetic profile of *CYP2C9, CYP2C19, CYP3A5* and *VKORC1* in Kosovo's population.

In the study that was conducted in Kosovo's healthy population for the first time, we have found a significant frequency of gene polymorphism of cytochrome P450 enzymes: CYP2C9, CYP2C19 and CYP3A5, as well as drug target VKORC1.

Such enzymes' polymorphism data might be used in dose adjustment of a number of clinically important drugs for preventing and minimizing adverse drug reactions, which sometimes may even cause death.

These polymorphisms can be very useful biomarkers in pharmacotherapy, especially in the individualisation of pharmacotherapy and avoidance of drug's side effects. In general, this study's data corresponds to the data from other white Caucasian populations but differs significantly compared to the data from populations from other continents.

2. This study did not find a significant difference between male and female participants regarding *CYPs* or *VKORC1* polymorphisms.

3. The frequency of *CYP2C9*1*1* genotype in this study differs compared to a number of European populations. However, on the other side, the majority of other genotypes of *CYP2C9* did not differ significantly in comparison to other white Caucasian populations. Regarding variant alleles **2* and **3*, their frequency is in accordance with the frequency of respective variant alleles in other European populations.

4. The analysis of *CYP2C19* polymorphisms revealed data that is similar to that from the neighboring countries such as Macedonia or Greece, but differs significantly when compared to Asians and Africans.

5. Within the analyzed *CYP* polymorphisms, the presence of *CYP3A5*3* gave us some interesting results. Its frequency was in accordance with that of Croats and Tuscans (Italy) as well as with residents of Utah (USA), but is different compared to data obtained from other Caucasians. There is a deeper difference in comparison with populations from other world areas such as Africa or Asia.

6. The latest gene that was characterized in this study was *VKORC1*. This study's findings regarding the *VKORC1* genotypes correspond to the data from Croats, but on the other side, we found difference when compared to data from Italians. No significant difference was observed with the data from Australia, both in the case of genotypes and variant alleles. Similar to other polymorphisms, there is a significant difference compared with Africans and Asians. With regard to variant alleles, there is no difference compared to Croats or Italians but there is a clear difference in comparison with Asians or Africans.
7. Finally, with respect to allelic variants, studies (including this study) have found and proved evident intraethnic variability apart from the interethnic differences. Therefore, Caucasians cannot be evaluated as a homogeneous group concerning *CYP2C9, CYP2C19, CYP3A5* and *VKORC1* variant frequencies.

## To whom it may concern

Knowing the distribution of these gene variants in Kosovo may prove very useful for the future research of certain complex diseases and for determining the impact of geographical and climatic conditions in their pathogenesis. Our data will form the basis for detecting the genetic risk factors related to specific diseases, including the toxic potential of numerous environmental pollutants. In addition, they can prove relevant to clinical pharmacokinetic studies and dosage recommendations for the Kosovo population.

# 8. ABSTRACT

## PhD thesis: 'GENETIC POLYMORPHISM OF CYP2C19, CYP2C9 AND VKORC1 IN KOSOVO POPULATION'

**Valon Krasniqi**

**2017**

**Background:** For the first time, we determined and analyzed frequency of the most important variant alleles of *CYP2C9, CYP2C19, CYP3A5* and *VKORC1* in Kosovo's population.

**Methods**: Determination of genetic polymorphism was conducted in 234 nonrelated Kosovars and genotyping was conducted by Real-Time PCR.

**Results**: Allele frequencies of *CYP 2C9*2* and *2C9*3* were 17,52% and 10.89% respectively. 16 subjects (6.81%) were anticipated to be poor metabolizers. For *CYP2C19, *2* and **17* variant frequencies were *2C19*2*=13.03% and *2C19*17*=19.01%. CYP2C19 poor metabolizers were predicted to be 2.13%, while 10 subjects (4.27%) were homozygous carriers of **17* allele and were predicted to be UM. For *CYP3A5*, prevalence of **3* variant allele in Kosovo population was 98.29 % (non-expressors). In relation to *VKORC1*, 33,3% of subjects of this study were found as homozygous (*CC*), 51,7% were heterozygous *CT* and 14.95% were homozygous for *T* allele.

**Discussion**: For *CYP2C9 and CYP2C19*, frequency of variant alleles is in accordance with respective data in Caucasians especially with data of Croats, Macedonians, Greeks and French people. For *CYP3A5*3*, its frequency was in accordance with Croats and Tuscans (Italy) but is different compared to data obtained from other Caucasians. Data on *VKORC1 (1173C>T)* correspond to the relevant genotypes of the Balkan populations such as Croats, but differ from other European countries such as Italy.

**Conclusion**: Findings of this study showed high accordance of variant alleles, genotypes and predicted phenotypes of Kosovars with that of other Caucasians, specifically with South Eastern European populations.

**Key words**: **pharmacogenetics; polymorphism; Kosovo; genotype; phenotype; variant allele; cytochrome P450 (CYP450); *CYP2C9*; *CYP2C19*; *CYP3A5*; *VKORC1*.**

# 9. SAŽETAK (EXPANDED ABSTRACT IN CROATIAN)

## Doktorska Disertacija: 'GENETIČKI POLIMORFIZAM CYP2C19, CYP2C9 I VKORC1 U KOSOVSKOJ POPULACIJI'

**Valon Krasniqi**

**2017**

**Pozadina**: Nema podataka o farmakogenetičkim polimorfizmima u populaciji Kosova.

Po prvi put smo odredili i analizirali učestalost najvažnijih varijantnih alela *CYP2C9, CYP2C19*, *CYP3A5* i *VKORC1* u populaciji Kosova.

**Metode**: Genotipizacija je provedena u 234 zdrava stanovnika Kosova u dobi od 18 do 65 godina. Svi sudionici su uključeni u ispitivanje nakon potpisivanja informiranog pristanka. Istraživanje je provedeno u krvno nepovezanih osoba koje predstavljaju mješovitu populaciju iz svih dijelova Kosova. Rodni omjer sudionika u istraživanju bio je gotovo jednak za žene i muškarce (116: 118). DNA je dobivena iz uzoraka krvi s antikoagulansom EDTA, uz korištenje komercijalnog kita QIAGEN® za ekstrakciju DNA. Genotipizacija je provedena metodom PCR u stvarnom vremenu (engl. *Real-Time PCR*) komercijalnim testovima *TaqMan® SNP* za genotipizaciju.

**Rezultati**: Učestalost alela *CYP2C9* *2 i *2C9* *3 je iznosila 17,52% i 10,89%. 16 ispitanika (6,81%) s genotipom *CYP2C9*2/*2, CYP2C9*2/*3* ili *CYP2C9*3/*3* pripada fenotipu slabih/sporih metabolizatora (PM). Određena je i učestalosta alela *CYP2C19*2* (13,03%) i *2C19*17* (19,01%). Ne temelju tih rezultata predviđa se učestalost CYP2C19 slabih metabolizatora (*CYP2C19 *2/*2* ) koja je iznosila 2,13%, dok je 71 ispitanik (30,33%) s genotipom *CYP2C19 *1/*17* ili *2C19*17/*17* klasificiran kao vrlobrzi metabolizator (UM). 10 ispitanika (4,27%) su bili homozigotni nositelji alela *17. Nadalje, 8 ispitanika (3,41%) nositelji su kombiniranog genotipa *CYP2C19*2/*17*. Za *CYP3A5*, prevalencija alela *3 bila je 98,29% (nonekspresori), dok je 1,7% ispitanika kategorizirano kao ekspresori. Zanimljivo je da genotip *CYP3A5*1/*3* nije detektiran u ovoj studiji. S obzirom na polimorfizam *VKORC1 1173C> T*, 33,3% ispitanika u ovoj studiji su bili homozigotni nositelji alela *C* (*1173CC*), 51,7% su bili heterozigoti (*1173CT*) a 14,95% homozigoti za alel *T* (*1173TT*).

**Rasprava**: Učestalost varijantnih alela *CYP2C9* je u skladu s prosječnom učestalosti ovih alela u nekim bjelačkim populacijama. Npr. naši podaci odgovaraju objavljenim podacima za hrvatsku i

francusku populaciju. Učestalost sporih metabolizatora putem CYP2C9, iznosila je 6,8%, što je blisko rezultatima za španjolsku populaciju (5%) i podudarno je podacima za susjedne zemlje Kosova kao što su Makedonija (p=0,3696), Grčka (p=0,2123) ili Hrvatska (p=0,0857), dok se značajno razlikuje od vrijednosti učestalosti polimorfizama objavljenih za Švedsku (p=0,0090), Ujedinjeno Kraljevstvo (p=0,0345) ili Rusiju (p=0,0077).

Učestalost varijantnih alela *CYP2C19* je usporediva s podacima za populaciju Makedonije i Grčke. Ustanovljena je značajna podudarnost u prevalenciji varijantnih alela i genotipova *CYP2C19* između Kosova s jedne strane i Makedonije (za *2C19*2* (p=055), i *2C19*17* (p=0,69) te Grčke (za *2C19*2* (p=1), i *2C19*17* (p=0,84). Naši nalazi također pokazuju značajnu razliku u usporedbi s ruskom populacijom (za *CYP2C19*1*1* p=0.0003). Ta razlika je još značajnija u usporedbi s vrijednostima za populacije drugih kontinenata kao što su kolumbijska (Južna Amerika) ili indijska (p < 0,0001). Učestalost *CYP3A5*3*, je u skladu s vrijednostima zabilježenima u populaciji Hrvatske, Toskane (Italija), Utaha (SAD), ali se razlikuje u usporedbi s podacima za neke druge bjelačke populacije. Zabilježena učestalost genotipova *VKORC1* 1173C> T u ovoj studiji odgovara relevantnim genotipovima balkanskih populacija kao što je hrvatska (1173 CC = 33,9%, CT = 46,8% i TT = 19,4%), dok se učestalost razlikuje u odnosu na neke europske zemlje, npr. Italija (*1173 CC* = 43,2%, *CT* = 43,9% i *TT* = 12,9%).

**Zaključci**: U studiji koja je po prvi put provedena na zdravom stanovništvu Kosova, otkrili smo značajnu učestalost polimorfiza gena enzima citokroma P450: *CYP2C9*, *CYP2C19* i *CYP3A5*, te ciljne molekule *VKORC1*. Nije ustanovljena značajna razlika u učestalosti polimorfizama u odnosu na spol.

Nalazi ove studije pokazali su visok stupanj sukladnosti učestalosti varijantnih alela, genotipova i predviđenih fenotipova Kosovara s onima drugih autohtonih europskih etničkih skupina, osobito s populacijama jugoistočne Europe. Najznačajnije su razlike zabilježene u odnosu na podatke za populacije drugih kontinenata.

**Ključne riječi: farmakogenetika; polimorfizam; Kosovo; genotip; fenotip; varijantni alel; Citokrom P450 (CYP); *CYP2C9*; *CYP2C19*; *CYP3A5*; *VKORC1*.**

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# 11. CURRICULUM VITAE

Valon Krasniqi is born on December 19, 1979 in Prishtina/Kosovo. He finished primary school and gimnasium in Prishtina. He graduated from the Faculty of Medicine, University of Prishtina, in 2005. In 2007, he was appointed a full-time Teaching Assistant of Pharmacology and Toxicology and Clinical Pharmacology at the Faculty of Medicine, University of Prishtina. In the period 2008-2013, he completed medical residency in Clinical Pharmacology.

**2012**: PhD thesis proposal (public) defence: "Genetic Polymorphism of CYP2C19, CYP2C9 and VKORC1 in Kosovo's population" at the School of Medicine/University of Zagreb, Croatia.
**2012**: Membership in ISPOR (Association for Pharmacoeconomy and Therapy Outcomes).
**2013**: Data Collection Team Leader on 'The evaluation of antibiotic prescription in the primary health care setting of Kosovo' project, organised by the WHO (World Health Organization) and the Ministry of Health of Kosovo.
**2013-2014**: Member of the 'Committee for Narcotic Drugs' within Kosovo Medicines Agency (KMA).
**2015**: Lecture at the '4$^{th}$ Pediatric School of the Republic of Kosovo'.
**2016**: Lecture at the '5$^{th}$ Pediatric School of the Republic of Kosovo'.
**2017**: Lecture at the '6$^{th}$ Pediatric School of the Republic of Kosovo'.
**2017**: Lectures on 'Pharmacogenetic analyses and their importance in clinical practice' on the 'Applicable Biology' Masters Program.

He has actively participated at congresses and continuing professional education (CPE) courses held in Kosovo and abroad.

He has published numerous works in medical journals.