

# The frequency of SLC01B1 c.388A>G and SLC01B1 c.521T>C polymorphisms in the Croatian population

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SVEUČILIŠTE U RIJECI

FAKULTET BIOTEHNOLOGIJE I RAZVOJA LIJEKOVA

Diplomski sveučilišni studij

„Biotehnologija u medicini“

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Učestalost polimorfizama *SLCO1B1* c.388A>G i *SLCO1B1* c.521T>C u  
hrvatskoj populaciji

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UNIVERSITY OF RIJEKA

FACULTY OF BIOTECHNOLOGY AND DRUG DEVELOPMENT

Masters program

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## Sažetak

Farmakogenetika igra ključnu ulogu u personaliziranoj medicini, posebice u optimizaciji učinkovitosti lijekova i smanjenju nuspojava. Gen *SLCO1B1*, koji kodira transportni protein SLCO1B1, važan je za unos statina u jetru, utječući na njihovu farmakokinetiku i farmakodinamiku. Ovo istraživanje istražuje učestalost polimorfizama *SLCO1B1* c.388A>G i *SLCO1B1* c.521T>C u hrvatskoj populaciji. TaqMan® metodom genotipizirano je ukupno 459 hrvatskih pacijenata. Rezultati su pokazali da je polimorfizam c.388A>G gotovo ravnomjerno raspoređen, s učestalošću alela od 52,96% za divlji tip (A) i 47,04% za varijantu (G). Nasuprot tome, polimorfizam c.521T>C bio je rjeđi, s učestalošću alela divljeg tipa (T) od 80,94% i učestalosti alela varijante (C) od 19,06%. Ovi su nalazi u skladu s prethodnim istraživanjima provedenim na hrvatskoj i europskoj populaciji. Daljnjom analizom istražen je odnos između ovih polimorfizama i demografskih čimbenika kao što su spol, dob i godina rođenja. Dok polimorfizam c.388A>G nije pokazao značajnu povezanost s ovim varijablama, polimorfizam c.521T>C pokazao je značajnu rodnu razliku u svojoj distribuciji, i to u heterozigotnom genotipu (521T/C). Dodatno, uočena je značajna korelacija između godine rođenja i homozigotne varijante genotipa (521C/C), što ukazuje na potencijalne generacijske promjene u frekvencijama alela. Identifikacija takvih genetskih varijanti u hrvatskoj populaciji naglašava važnost integracije farmakogenetskog testiranja u rutinsku kliničku praksu. Prilagodбом terapije statinima prema genetskom profilu pojedinca, pružatelji zdravstvenih usluga mogu optimizirati učinkovitost liječenja dok minimaliziraju rizik od nuspojava lijekova. Ovaj pristup ne samo da poboljšava skrb za pacijente, već ima i šire implikacije na javno zdravstvo, potencijalno dovodeći do učinkovitijih i personaliziranih strategija liječenja u Hrvatskoj i šire. Kako se razumijevanje farmakogenomike nastavlja razvijati, studije poput ove doprinose sve većem broju dokaza koji podržavaju kliničku korisnost

genetskog testiranja u optimizaciji terapije lijekovima i unapređenju personalizirane zdravstvene skrbi.

Ključne riječi: *farmakogenetika, transportni proteini, SLCO1B1, genski polimorfizam, hrvatska populacija*

## Summary

Pharmacogenetics plays a critical role in personalizing medicine, particularly in optimizing drug efficacy and minimizing adverse effects. The *SLCO1B1* gene, encoding the SLCO1B1 transporter, is important for the hepatic uptake of statins, influencing their pharmacokinetics and pharmacodynamics. This study investigates the frequency of *SLCO1B1* c.388A>G and *SLCO1B1* c.521T>C polymorphisms in the Croatian population. A total of 459 individuals of Croatian descent were genotyped using the TaqMan® method. The results revealed that the c.388A>G polymorphism is nearly evenly distributed, with allele frequencies of 52.96% for the wild-type (A) and 47.04% for the variant (G). In contrast, the c.521T>C polymorphism was less common, with a wild-type allele (T) frequency of 80.94% and a variant allele (C) frequency of 19.06%. These findings align with previous studies conducted in both Croatian and European populations. Further analysis explored the relationship between these polymorphisms and demographic factors such as gender, age, and year of birth. While the c.388A>G polymorphism showed no significant associations with these variables, the c.521T>C polymorphism demonstrated a notable gender difference in its distribution in the heterozygous genotype (521T/C). Additionally, a significant correlation was observed between the year of birth and the homozygous variant genotype (521C/C), suggesting potential generational shifts in allele frequencies. The identification of such genetic variants in the Croatian population underscores the importance of integrating pharmacogenetic testing into routine clinical practice. By tailoring statin therapy to an individual's genetic profile, healthcare providers can optimize treatment efficacy while minimizing the risk of adverse drug reactions. This approach not only enhances patient care but also has broader implications for public health, potentially leading to more effective and personalized treatment strategies in Croatia and beyond. As the understanding of pharmacogenomics continues to evolve, studies like this contribute to the growing body of



evidence supporting the clinical utility of genetic testing in optimizing drug therapy and advancing personalized healthcare.

Key words: *pharmacogenetics, drug transporter, SLCO1B1, gene polymorphism, Croatian population*

## **Abbreviations**

SNP – single nucleotide polymorphism

SNV – single nucleotide variant

DPD – dihydropyrimidine dehydrogenase

EMA – European Medicines Agency

CPIC – Clinical Pharmacogenetics Implementation Consortium

TPMT – thiopurine S-methyltransferase

*wt* – wild type

CYP – cytochrome P450

PM – poor metabolizer

IM – intermediate metabolizer

NM – normal metabolizer

VKORC1 – Vitamin K Epoxide Reductase Complex subunit 1

HMG-CoA – 3-hydroxy-3-methylglutaryl-coenzyme A

LDL – low-density lipoprotein

PCSK9 – proprotein convertase subtilisin/kexin type 9

SAMS – statin-associated musculoskeletal symptoms

SIM – statin-induced myopathy

CK – creatine kinase

ATP – adenosine triphosphate

ABCB1 – ATP-binding cassette subfamily B member 1

UGT – uridine 5'-diphospho-glucuronosyltransferase

SLCO1B1 – solute carrier organic anion transporter family member 1B1

ABC – ATP-binding cassette

SLC – solute carrier

AUC – area under the curve

ADRs – adverse drug reactions

GWAS – genome-wide association study

OR – odds ratio

CI – confidence interval

*mut* – mutant

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# 1. Introduction

## 1.1. Pharmacogenetics

Pharmacogenetics is the study of genetic variation in pharmacological response, according to one definition. "Pharmacogenomics", however, as a phrase, has lately gained more popularity in the world of science. While the former phrase is mostly used in reference to genes regulating drug metabolism, the latter is a more generic term that encompasses all genes in the genome that may determine drug response. Both names, however, can be used interchangeably and the distinction is arbitrary. Still, numerous studies on pharmacogenomics have been published in many journals over the past 12 to 18 months. This is due to the fact that pharmacogenomics is thought to be a very essential field for future medication therapy and prescription improvement.(1,2) Only with time will it be clear if and to what extent this prediction is true, though there are already some examples of success.(3-5)

About every 500-1000 nucleotides, the human genome exhibits variation. Out of these, single nucleotide polymorphisms (SNPs) have drawn the greatest attention despite the fact that there are many other types of polymorphic markers because of their potential to be utilized to determine an individual's specific medication response profile.(1) When a single nucleotide is changed for another at a specific location in a person's genome, it is classified as either an SNP or a single nucleotide variant (SNV); the distinction is that SNPs happen more frequently than SNVs in the population (they are present in at least 1% of the population, or higher). These variants are frequently denoted by an asterisk and a number that corresponds to the particular mutation present in that allele (e.g., *CYP450 2D6\*4*). (2) A collaboration between nonprofits such as the Wellcome Trust and the pharmaceutical sector produced a library of 300,000 SNPs. The most recent outcome of this effort, which was consistently finished considerably ahead of time, was the publication of an SNP map with 1.42

million SNPs at an average density of one SNP every 1.9 kilobases.(6) In theory, this may be utilized to produce distinct SNP profiles that correspond with distinct drug reactions. The current approach of administering medication follows the principle that "one dose fits all." Customizing a patient's medication prescription and dosage could maximize efficacy and minimize toxicity, thanks to SNP profiling.(3,5) Since personalized medicines hold the potential to streamline the drug development, testing, and registration processes and reduce the time from chemical synthesis to introduction into clinical practice and, thus, the cost of the process, the pharmaceutical industry is clearly interested in and concerned about the promise of personalized medicines.(1,7)

## 1.2. Pharmacogenetics and drugs

The purpose of pharmacogenomics is to apply genetics to improve pharmacological therapy, boost therapeutic efficacy, and lessen adverse drug reactions. The application of pharmacogenomics in clinical practice lags behind this knowledge despite the fact that a direct correlation exists between genetic predisposition and the efficacy and toxicity of specific medications. However, there are a few notable instances of pharmacogenetic tests used successfully in clinical practice.(7,8)

Pharmacogenetics is mostly applied in oncology, with the goal of improving patient care standards. The gene DPYD, encoding dihydropyrimidine dehydrogenase (DPD), which is a rate-limiting enzyme for the catabolism of fluoropyrimidines, is a notable example of this. It is known that there are numerous genetic variants in DPYD that change the mRNA splicing or protein sequence; some of these variants lead to decreased enzyme function. A considerable segment of the populace lacks DPD, an enzyme required for the metabolism of fluorouracil and other similar medications. Fluorouracil can therefore accumulate in their blood after treatment with these medications, which can result in serious and potentially fatal side

effects like neurotoxicity (damage to the nervous system), stomatitis (inflammation of the mouth lining), neutropenia (low levels of neutrophils, a type of white blood cells needed to fight infection), and severe diarrhea. Four decreased function DPYD variations are of primary interest in the context of 5-fluorouracil, a medication commonly used in the treatment of solid malignancies, because of their population frequency and proven influence on enzyme function and toxicity risk; c.1905+1G>A (\*2A) is the most well-studied variant. Considering this, it has been found that patients with decreased/no function variations (heterozygous for DPYD) exhibit partial DPD deficiency and, as a result, should have lower starting doses. Furthermore, a 50% dose reduction after prospective genotyping of c.1905+1G>A in heterozygous carriers led to a risk of severe toxicity equivalent to that of non-carriers. Thus, this study showed that 50% dosage reduction is appropriate for heterozygous carriers of no function variations and that DPYD genetic testing can lower the incidence of severe fluoropyrimidine-related toxicity. Naturally, these and other findings can contribute to the development of guidelines that represent the consensus of experts and are based on clinical evidence and peer-reviewed literature that is available at the time of writing. Therefore, similar recommendations regarding testing and treatment have been released by the European Medicines Agency (EMA) and the Clinical Pharmacogenetics Implementation Consortium (CPIC). These recommendations primarily state that patients with partial deficiency may start cancer treatment at lower doses than usual and that patients who completely lack DPD must not be given any fluorouracil medicines.(4,5) Still, these suggestions are intended only to assist clinicians in decision making and to identify questions for further research as new evidence emerges.(4) Hopefully, in the future, they become more akin to rules and standard procedures so that they may improve oncological and other therapies.

Another well-known example of a polymorphic enzyme involved in drug metabolism and its therapeutically relevant substrates is thiopurine S-



methyltransferase (TPMT; commonly referred to as thiopurines). Thiopurines, which include mercaptopurine, thioguanine, and azathioprine, are prescribed for autoimmune disorders, inflammatory bowel disease, and acute lymphoblastic leukemia. Many people with two wild type (*wt*) copies of the TPMT gene have low risk of thiopurine toxicity and can be treated with a standard dose of the drug (fast metabolizer). On the other hand, patients with lower TPMT levels are heterozygous for one *wt* gene and one genetic variation. About 30–60% of heterozygous individuals experience severe side effects from taking thiopurines at regular levels, necessitating either lowered dosages or the use of other medications. Those who are homozygous for the mutant *TPMT* gene and have little to no TPMT would definitely experience significant bone marrow toxicity (myelosuppression) when given standard doses of thiopurines; they will need to be treated with an other drug. Implementing TPMT testing in clinical practice has been shown to prevent severe toxicity and improve treatment outcomes in patients receiving thiopurine therapy.(9)

More relevant to this study, pharmacogenetics also has been more used in clinical settings during the past ten years to help select cardiovascular medicines. A famous example of this would be with the drug clopidogrel and the cytochrome P450 (CYP) 2C19 enzyme. A two-step metabolism converts the prodrug clopidogrel into its active form, which inhibits platelet activity permanently. Clopidogrel is activated by a number of CYP enzymes. Nevertheless, both stages include the highly polymorphic enzyme CYP2C19, which plays a critical role in the bioactivation of clopidogrel. People who carry two loss-of-function (no-function) alleles are poor metabolizers (PMs) and lack CYP2C19 enzyme activity. Intermediate metabolizers (IMs) have significantly lower enzyme activity and one no-function allele. It's been estimated that about 30% of people are PMs or IMs. Compared to the normal metabolizer (NM) phenotype, both of these phenotypes are linked to increased on-treatment platelet reactivity and decreased exposure to the active clopidogrel metabolite. The technique of using greater dosages of

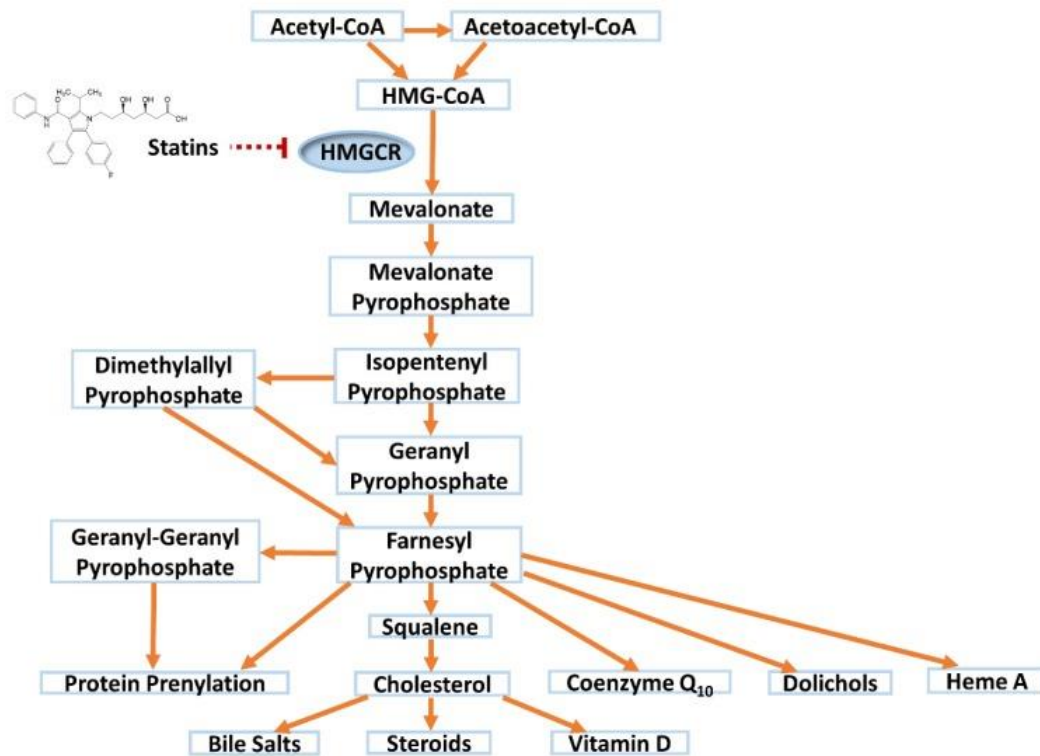
clopidogrel to compensate for the decreased antiplatelet effects in PMs and IMs has been studied. For IMs, tripling the dose to 225 mg daily can achieve the same amount of platelet inhibition as a 75 mg daily dose for NMs; however, even a dose of 300 mg daily is not enough for PMs. In the absence of contraindications (such as a significant risk of bleeding), ticagrelor and prasugrel are preferred over clopidogrel dose escalation in IMs and PMs since the *CYP2C19* genotype has no effect on the pharmacokinetics or pharmacodynamics of these medications.(10)

Another classic case is one of warfarin. It inhibits Vitamin K Epoxide Reductase Complex subunit 1 (VKORC1), making less Vitamin K accessible to function as a cofactor for clotting proteins. This makes it one of the anticoagulant drugs that is prescribed the most frequently worldwide. Warfarin, although effective, has a significant risk of both severe and small bleeding because of its narrow therapeutic index and large inter-individual variability in the appropriate dose (0.6–15.5 mg/day). For the purpose of administering warfarin and preventing adverse effects, the three most significant polymorphisms that have been shown to have clinical implications are the G-1639A polymorphism (*rs9923231*) of VKORC1 and the \*2 (*rs1799853*) and \*3 (*rs1057910*) alleles of *CYP2C9*. The VKORC1 G-1639A polymorphism is associated with lower warfarin dosage requirements in patients who are Caucasian and Asian. Individuals with the *CYP2C9*\*2 and \*3 variants are more likely to need lower doses of warfarin when commencing treatment, require more time to reach the target international normalized ratio, and are more prone to experience bleeding issues.(10,11)

These examples show the overarching goal of pharmacogenomics: to use genetic information to guide drug therapy, improving outcomes and reducing the incidence of adverse drug reactions across various medical fields, including oncology, cardiovascular medicine, and beyond. Another field where pharmacogenomics has proven useful is with statins, which will be covered now.

### 1.3. Statins

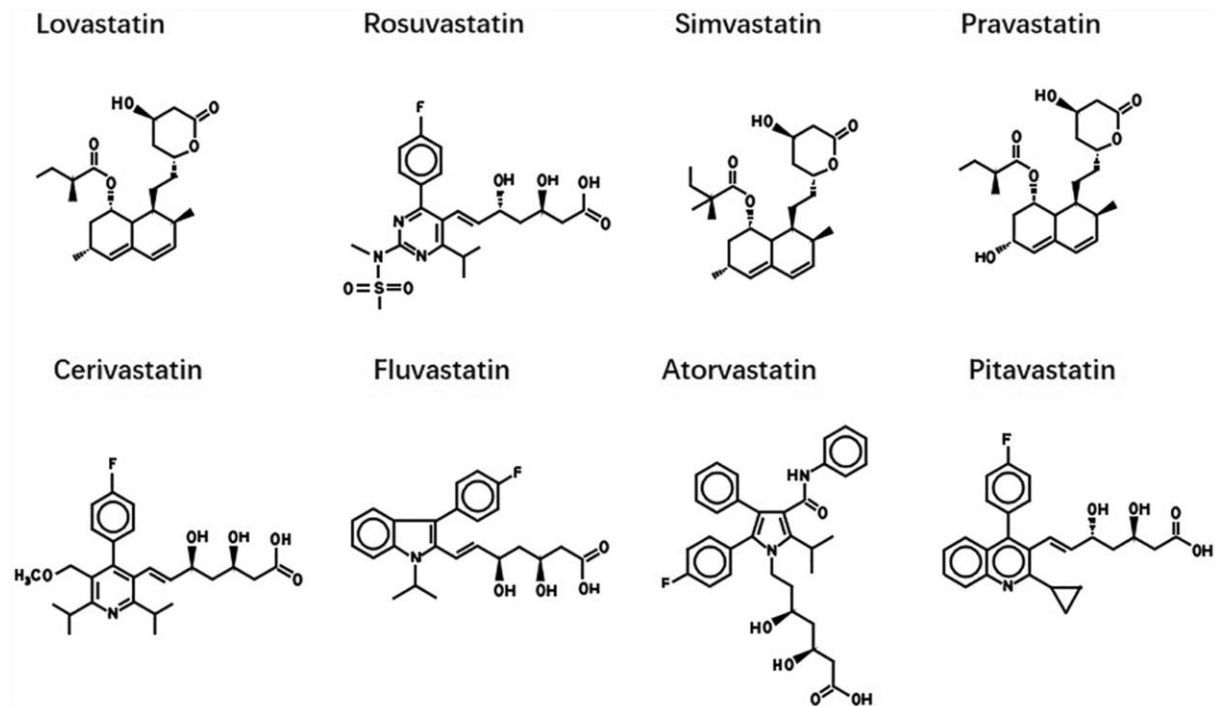
Human plasma cholesterol is produced by cells either through *de novo* biosynthesis or by food consumption. Statins are frequently used as lipid-lowering medications because they competitively block the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, limiting the synthesis of endogenous cholesterol.(12–14) Because of their structural similarity to HMG-CoA, they are able to fit into the active site of the enzyme and compete with it for the native substrate. This competition slows down the rate at which HMG-CoA reductase can produce mevalonate, the next molecule in the mevalonate pathway chain that eventually produces cholesterol (Figure 1). Stated differently, statins lower plasma cholesterol levels via altering the expression of the low-density lipoprotein (LDL) receptor and decreasing the production of new cholesterol.(15) The original intent of its development was to prevent cardiovascular disease by lowering cholesterol levels. The impact of statins on cardiovascular morbidity and mortality in patients with and without atherosclerotic disease has been established. Nevertheless, aside from its ability to decrease cholesterol, statins also seem to have pleiotropic effects. These effects include those on diabetes, cancer, inflammation, neurological disorders, and coronary heart disease.(13) In the course of the 1970s, statins were first developed as "cholesterol-lowering" medications. All three of the fungal-derived substances simvastatin, lovastatin, and pravastatin have fairly similar structures. However, pravastatin is more hydrophilic than simvastatin or lovastatin due to the presence of extra hydroxyl groups. Contrarily, the synthetic derivative drugs fluvastatin, pitavastatin, cerivastatin, atorvastatin, and rosuvastatin share structural commonalities, such as shared fluoride side groups (Figure 2). The chemical makeup of statins may affect their intracellular actions differently. Compared to hydrophilic statins (pravastatin and rosuvastatin), simvastatin has a greater ability to cross cell membranes and enter hepatocytes and non-hepatocytes through passive diffusion.(13,14)



**Figure 1.** Statin inhibition of the mevalonate pathway. *Adopted from Statin-Related Myotoxicity: A Comprehensive Review of Pharmacokinetic, Pharmacogenomic and Muscle Components, 2020.*(14)

Even if more recent proprotein convertase subtilisin/kexin type 9 (PCSK9) serine protease inhibitors can also be used to reduce serum cholesterol levels, statins are still often utilized in clinical treatment because they are well tolerated and improve the condition of patients with cardiovascular disease. However, continuous statin use can lead to a variety of statin-associated musculoskeletal symptoms (SAMSs), including myalgia, rhabdomyolysis, and life-threatening statin-induced myopathy (SIM).(12,16) Acute or persistent muscle soreness, myasthenia, and increased creatine kinase (CK) values in asymptomatic persons are some of the clinical symptoms of SIM. Some possible pathogenic mechanisms that may underlie the development of SIM include the lack of cholesterol, the decreased stability and permeability of the myocyte membrane, the lack of coenzyme Q10, which causes dysfunctional mitochondrial respiration and energy production in myocytes, and the decreased isozyme synthesis, which increases the risk of muscle toxicity. However, medication tolerance, underlying medical disorders, and genetic factors all affect how frequently

SIM occurs in a given person. Every 1000 patients taking statins experience SIM, on average. As a result, SIM is the primary cause of statin therapy non-adherence and/or cessation, which has negative cardiovascular effects.(12)



**Figure 2.** Chemical structures of selected statin. Adopted from *Statins: a repurposed drug to fight cancer*, 2021.(13)

The genetic components of SIM have been the subject of extensive research. Particularly, it has been observed that statin pharmacokinetics, and SIM incidence to an extent, are impacted by SNVs in several genes. These are the efflux transporters ABCB1 (adenosine triphosphate (ATP)-binding cassette subfamily B member 1) and ABCG2(17), CYPs(17), UGTs (uridine 5'-diphospho-glucuronosyltransferases), and SLCO1B1 (solute carrier organic anion transporter family member 1B1). These SNVs generally lead to reduced protein levels and higher statin exposure. Only SLCO1B1, a crucial statin transporter involved in drug detoxification in the

liver, has been consistently linked to SIM out of all the statin pharmacokinetics genes examined, and as a result, it is the focus of extensive research.(12,14)

#### 1.4. Transporter molecules

Although a drug molecule can move by simple diffusion, cell membranes have a variety of transporter proteins that help with efflux or influx via active transport.(18) The mechanism of transport is a common classification scheme for transport proteins. ATP-binding cassette (ABC) transporters are the main active transporters found in higher species. They propel solutes across membranes and usually out of cells by hydrolyzing ATP. Secondary active transporters link solute translocation to inorganic ion co-transport or counter-transport of other solutes; in the latter instance, they are frequently referred to as exchangers. Facilitative transporters, on the other hand, translocate solutes across the membrane according to their electrochemical gradient. The varied and heterogeneous solute carrier (SLC) superfamily of proteins represents secondary and facilitative transporters in humans and higher species. All transmembrane proteins that facilitate the facilitative or secondary active translocation of solutes across the membrane, such as nutrients, metabolites, ions, and xenobiotics, are classified as SLC solute carriers. Transporter proteins constitute a vital protein mechanism that works in tandem with the ATP-dependent ABC transporters to regulate the cellular and systemic homeostasis of all solutes in our body and to maintain the required ion gradients.(19)

About 11 ABC transporters and about 26 SLC transporters are believed to be directly involved in drug translocation out of the 48 proteins that make up the ABC and over 400 proteins that make up the SLC transporter superfamilies in humans. Understanding the distribution of transporter proteins in different human organs is essential to comprehending their function in drug metabolism and applicability as targets for drug

administration. The presence of transporters in the intestine, liver, and kidney—the main organs that determine the absorption, distribution, metabolism, excretion, and toxicity of drugs—as well as in the blood-brain barrier, which controls the entry of central nervous system drugs into the brain, have been the subject of extensive research since their significance in pharmacokinetics was first recognized many years ago. Currently, a large number of these ABC and SLC transporters are identified in the plasma membranes of kidney, liver, and biological barrier cells. As a result, they have a significant impact on the pharmacokinetics of small molecule medications in the body. In addition to investigating how current medications interact with transporters, strategies have been devised to take use of the distinct cellular localization and transport function of transporters as entry points for the delivery of medications to certain organs and through particular biological barriers.(19)

Transporters have distinct expression patterns and transport efficiencies due to the polymorphism of the genes encoding them. Thus, frequent variations in the genes that code for transport proteins lead to variations in the pharmacokinetics of drugs and, ultimately, in the response of the patient to treatment. A number of these genetic variations lead to changes in translational efficiency (e.g., differences in mRNA folding), mRNA expression levels (e.g., promoter variants), and protein function (e.g., coding polymorphisms). Variations in clinical endpoints such as toxicity and response are ultimately caused by interindividual variability in drug disposition, which is largely explained by genetic variability in transporters. In order to finally customize treatment with substrate medications based on genotype, the study of transporter pharmacogenetics aims to clarify the mechanisms by which genetic diversity in transporters produces individual variances in drug transport. (18)

For the last 20 years, the link between the genotype and phenotype of transporter genes has been the subject of much study.(7) The ultimate objective of transporter pharmacogenetics research is to deepen our

comprehension of the manner in which transporter genetics impacts clinical outcomes, with the aim of improving the safety and effectiveness of present medication treatments as well as the development of novel therapeutics. Before changing any medication, it is crucial to conduct powerful and prospective studies to evaluate the true impact of these polymorphisms and identify if they are caused by the disease or the drug.(18) More studies have been conducted recently evaluating genes encoding solute carriers that mediate the cellular uptake of drugs, such as *SLCO1B1* and *SLC22A1* (*OCT1*), even though most transporter-related pharmacogenetic research has been in relation to classic genes encoding the outward-directed ATP-binding cassette transporters, such as *ABCB1* (P-glycoprotein), *ABCC2* (*MRP2*), and *ABCG2* (*BCRP*). Pharmacokinetically important tissues such as the intestine (drug absorption), blood-brain barrier (distribution), liver (metabolism), and kidneys (excretion) all have ABC and SLC transporters, which suggests that genetic variation related to changes in these transporters' protein expression or function may have a major impact on systemic drug exposure and toxicity.(20)

### 1.5. *SLCO1B1*

A key hepatic influx transport protein, *SLCO1B1* (solute carrier organic anion transporter family member 1B1) (other protein names include *OATP1B1* and *OATP-C*), is produced by the *SLCO1B1* gene.(3,16,21) This drug transporter, which is most commonly expressed in the liver, aids in the hepatic absorption of a wide range of clinically used medications, such as endothelin receptor antagonists (bosentan), angiotensin-converting enzyme inhibitors (enalapril, temocapril), methotrexate, angiotensin II receptor blockers (valsartan, olmesartan), plus a variety of endogenous substances (bile acids, thyroid hormones, bilirubin, estrone-3-sulfate, and estradiol-17 $\beta$ -glucuronide).(16) Clinically relevant drug substrates include hypolipidemics (atorvastatin, pravastatin, rosuvastatin, ezetimibe),



protease inhibitors (darunavir, lopinavir), cytostatics (SN-38, pazopanib) and antibacterial drugs (benzylpenicillin, cefditoren, rifampicin).(22)

The *SLCO1B1* gene, which is found on the short arm of chromosome 12, spans 109 kilobases (kb) and has 15 exons. The gene locus contains the 609 kb long, closely linked *SLCO1B1*, *SLCO1B3*, *SLCO1B7*, and *SLCO1A2* genes. While *SLCO1A2* is encoded on the negative strand, the three members of the *SLCO1B* family are encoded on the positive strand. It should be noted that while exon 2 contains the ATG start codon, exon 1 is still untranslated and belongs to the 5' untranslated region. Despite the fact that this gene has many SNVs, only a small number of them are known to have clinically significant functional effects.(3)

Numerous *in vitro* and *in vivo* studies have revealed that genetic diversity can affect *SLCO1B1* function in a variety of ways. While some alleles (*SLCO1B1*\*5, \*15, \*23, \*31, and \*46-\*49) are linked to decreased function, others (*SLCO1B1*\*14 and \*20) are linked to increased function as a result of higher expression (see PharmGKB allele functionality table(23)). The effects of these alleles may differ depending on the substrate. It is well known that the *SLCO1B1* gene varies greatly between and within populations (see below and PharmGKB allele frequency table(23)). Although genetic variation has a significant impact on function, other variables, such as drugs that block *SLCO1B1*, may also affect how variable *SLCO1B1* function is.(16) The most extensively studied variants are *rs2306283* (c.388A>G, p.N130D) and *rs4149056* (c.521T>C, p.V174A) among papers detailing *SLCO1B1* variant and allele frequencies across populations. There are four significant star alleles/haplotypes as a result: *SLCO1B1*\*1 (formerly \*1A), containing neither variant, *SLCO1B1*\*37 (formerly \*1B), containing *rs2306283*, *SLCO1B1*\*5, containing *rs4149056*, and *SLCO1B1*\*15, containing both (Table 1).(16,21)

The predicted frequencies for specific alleles vary greatly both within and within the biogeographical categories. While the allele frequencies recorded

for *SLCO1B1*\*15 range from 24% in American, 15% in European, 12% in East Asian, 15% in Near Eastern, 7% in Central/South Asian, and 3% in African Oceanian populations, those reported for *SLCO1B1*\*5 range from 2% in European and 1% in other populations. The frequency of *SLCO1B1*\*37 varies substantially between populations; among African and Asian populations, it is significantly more common (75%) and significantly less common (40%). Numerous alleles have insufficient frequency data, and the tabulated allele frequencies—including those for *SLCO1B1*\*37—may be inaccurate as a result of the short sample size, insufficient variant testing, and/or other factors. Because there may be significant variations in allele frequencies within each biogeographical group, the combined allele frequencies for each biogeographical population are estimates.(16)

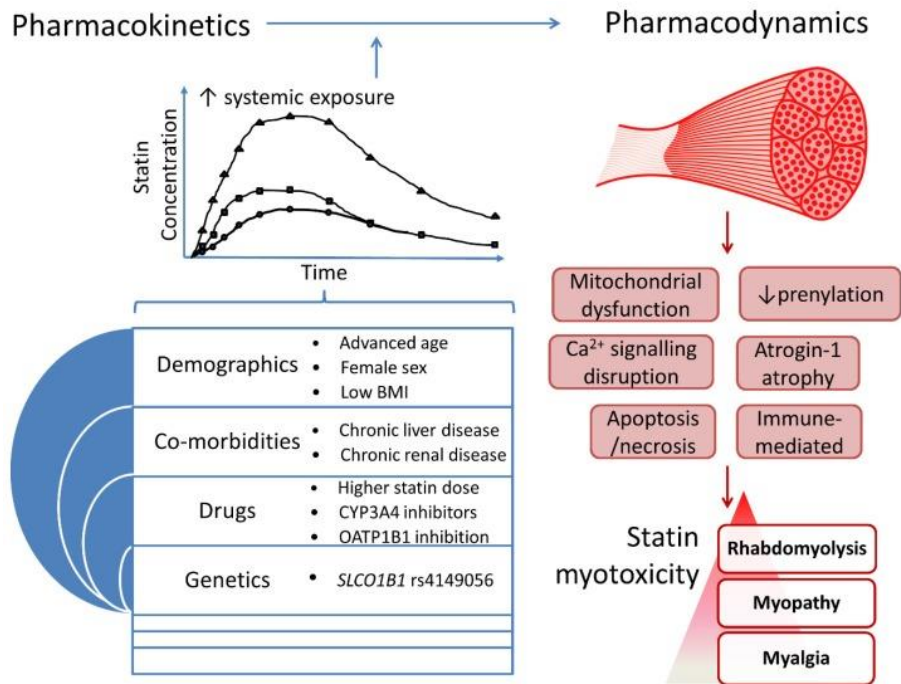
**Table 1.** Relevant *SLCO1B1* alleles and their statuses.(23)

Allele	Allele Clinical Functional Status	Strength of Evidence	Summary of Findings
*1	Normal function	Definitive	reference allele, normal <i>SLCO1B1</i> activity
*37	Normal function	Strong	normal function based on strong evidence in heterozygous and homozygous individuals, plasma levels of highly sensitive <i>SLCO1B1</i> endogenous substrates are slightly and non-significantly lower, <i>in vitro</i> studies have mostly demonstrated unaltered activity compared to <i>SLCO1B1</i> *1
*5	No function	Definitive	no function based on definitive evidence in heterozygous and <i>SLCO1B1</i> *5/*15 compound heterozygote individuals, plasma levels of highly sensitive <i>SLCO1B1</i> endogenous substrates are significantly higher in <i>SLCO1B1</i> *1/*5 and <i>SLCO1B1</i> *5/*15 heterozygotes than in individuals with the <i>SLCO1B1</i> *1/*1 homozygotes, <i>in vitro</i> studies have demonstrated significantly reduced uptake of estrone sulfate and estradiol 17beta-d-glucuronide, and several other substrates compared to <i>SLCO1B1</i> *1
*15	No function	Definitive	no function based on strong evidence in heterozygous and homozygous patients, and <i>in vitro</i> studies, association with impaired function compared to <i>wild type</i> is well-established based on 15 years of research

## 1.6. SLCO1B1 and statins

Although a number of SIM risk factors have been found and potential processes have been put forth, there is still no common pathophysiological understanding of them. Nevertheless, two interrelated pathways are mentioned: intracellular skeletal myocyte entry and impairment of muscle function; and increased statin systemic exposure resulting from clinical and pharmacogenomic factors, which increase skeletal muscle exposure (Figure 3).

Increased exposure to statins is linked to some, but not all, of the clinical risk factors for SIM that have been discovered. For instance, taking more medication exposes one to statins, although this may not always translate to or be directly correlated with SIM. Except for fluvastatin and rosuvastatin, there is a slight positive correlation between increasing age and statin exposure. Additionally, with the exception of rosuvastatin and atorvastatin, women are typically exposed to most statins at somewhat higher rates. Women have also been shown to have somewhat lower circulating atorvastatin levels than males, which can be linked to increased hydroxylation metabolism, even if there is no difference in mean rosuvastatin exposure between the sexes. Aside from that, a lower body mass index, hepatic illness, and renal impairment are additional risk factors. In addition to these, a number of genes affect the pharmacokinetics of statins. But only *SLCO1B1*, more precisely the *rs4149056* variation, has been reliably linked to SIM.(14)



**Figure 3.** An in-depth look of the mechanisms responsible for statin myotoxicity. Adopted from *Statin-Related Myotoxicity: A Comprehensive Review of Pharmacokinetic, Pharmacogenomic and Muscle Components*, 2020.(14)

### 1.6.1. The rs2306283 variant

The c.388A>G (*rs2306283*) variant of the *SLCO1B1* gene hasn't seemingly been researched as much as the c.521T>C variant. It's often excluded from research papers pertaining to *SLCO1B1* in favor of the *rs4149056* variant, and even when it is explored, the data obtained is usually inconclusive (12,24–28). In the past, it was thought that this variant increases transporter function but these reports were inconsistent.(21) However, since then it has been mostly proven that it has normal function (see PharmGKB allele functionality table(23)), but there still appears to be some conflicts. With atorvastatin, some studies show that the genotype 388G/G is associated with increased reduction in LDL(29) in people with hypercholesterolemia as compared to genotypes 388A/A + 388A/G, and that allele G is associated with decreased plasma concentrations of atorvastatin as compared to allele A.(30) Others have found that the *wt*

(388A/A) is associated with increased response to atorvastatin in people with hypercholesterolemia as compared to genotypes 388A/G + 388G/G.(31) With pitavastatin, one study suggests that genotypes 388A/G + 388G/G are associated with increased pitavastatin plasma concentrations (area under the curve, AUC) when exposed in healthy individuals as compared to the *wt.*(32) Similar conflicting evidence could be found for pravastatin.(33,34) For rosuvastatin, the polymorphism correlated with decreased AUC.(35) Still, none of these studies researched the connection between the *rs2306283* variant and SIM, though a separate SEARCH study showed a link to reduced risk for simvastatin-associated myopathy.(28) The 388A>G polymorphism and adverse drug reactions (ADRs) brought on by different statins did not significantly correlate, according to a 2016 meta-analysis of nine cohort and four case-control studies comprising 11246 statin users, 2355 of whom developed ADRs.(36) Hopefully more research about this will be conducted to help better understand this variant and its function.

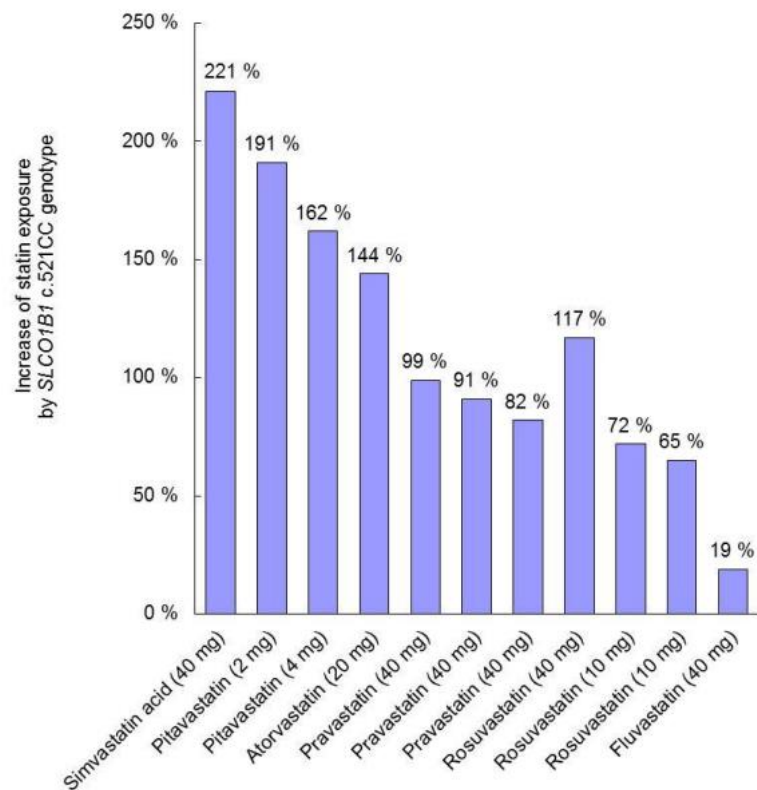
#### *1.6.2. The rs4149056 variant*

PharmGKB has deemed the *SLCO1B1*\*5 haplotype as a “no function” one (see PharmGKB allele functionality table(23)). This is naturally backed by numerous studies, like the ones where the *rs4149056* 521C/C homozygosity has been associated with increases in statin AUC of 286% for lovastatin acid(37), 221% for simvastatin acid(27), 208% for pitavastatin(38), 144% for atorvastatin(24), 91% for pravastatin(26), and 65% for rosuvastatin(24). A genome-wide association study (GWAS) revealed that, notably, *rs4149056* was substantially associated with myopathy in 85 cases compared to 90 controls, all of whom took 80 mg of simvastatin daily. The odds ratio (OR) for myopathy in those with 521C/C versus 521T/T genotypes was 16.9 (95% confidence interval [CI] 4.7–61.1), and a gene-dose trend was observed with an OR of 4.5 (95% CI 2.6–7.7) per C allele. Individuals taking 40 mg of simvastatin daily nevertheless

had a relative risk of 2.6 (95% CI 1.3-5.0) for myopathy per C allele, which is consistent with a medication side effect related to dosage.(28) Simvastatin myopathy and *rs4149056* have been therefore linked, and this association has been reproduced and confirmed in recent sizable meta-analyses and GWASs(39). Additionally, *rs4149056* has been connected to less severe negative effects like myalgia, prescription reductions, and/or small biochemical increases (such as CK) suggestive of simvastatin intolerance.(14) In addition to simvastatin, historical cases of cerivastatin-related rhabdomyolysis have been associated with *rs4149056*.(40) Further evidence of a connection between *rs4149056* and rosuvastatin myotoxicity came from recent meta-analyses(35,41) and an even more recent case-control study.(42)

It has been suggested that *rs4149056* may be relevant for severe myopathy caused by a number of statins, with an effect size likely greatest for simvastatin (or lovastatin) and lowest for fluvastatin (Figure 4). This is based on how much the *rs4149056* minor C allele increases exposure to each statin.(25) However, *rs4149056* has not yet been firmly linked to pravastatin myotoxicity, and while an association between *rs4149056* and atorvastatin myotoxicity has been hypothesized and published, multiple other studies found no evidence for it.(25,41). As atorvastatin appears less intrinsically myotoxic than simvastatin, there are fewer atorvastatin cases in studies (especially cases on high dose atorvastatin) and the impact of *rs4149056* on exposure is smaller for atorvastatin acid than simvastatin acid. These factors contribute to the ongoing uncertainty surrounding the role of *rs4149056* in atorvastatin myotoxicity(14), which is conceivable since atorvastatin uses *SLCO1B1*, *OATP1B3*, *2B1*, and *1A2* for hepatocyte absorption. A substantial correlation was also discovered in a 2016 meta-analysis between the *SLCO1B1* 521T>C polymorphism and a higher incidence of ADRs in simvastatin users, but not in atorvastatin users.(36) In 2024 however, a GWAS study did correlate the polymorphism with increased AUC of atorvastatin.(43) In conclusion, the impact of *rs4149056*

on the risk of myotoxicity is evident for simvastatin, but it is not fully understood for the other approved statins.(24)



**Figure 4.** Effect of the *SLCO1B1* c.521T/C variant on plasma exposure for different statins, CC vs. TT. Adopted from *The Clinical Pharmacogenetics Implementation Consortium Guideline for SLCO1B1 and Simvastatin-Induced Myopathy: 2014 Update*, 2014(44)

### 1.6.3. The CPIC guidelines regarding *SLCO1B1*

Pharmacogenetic test findings may offer extra helpful information for patients who are eligible for new statin therapy, given the balance between the established cardiovascular disease benefit and the risk of SAMS. Pharmacogenetic test findings may be used to switch to a different statin type or dose for patients currently receiving statin medication, depending on how long the patient has been tolerating the statin. For individuals who have a diagnosis of *SLCO1B1* genotype, statin therapy should not be



avoided or stopped based on the results, especially if the patient and provider are involved in the decision-making process.(3)

Simvastatin therapy and *SLCO1B1* were the subjects of the first CPIC guidelines, which were released in 2012(45) and 2014(44). A revised guideline covering more statin medications was released in 2022(3). These recommendations summarize the corpus of current research and offer therapeutic guidance for prescription statins depending on the *SLCO1B1* genotype. PharmGKB and CPIC worked together to establish standard format files with allele definition mapping, allele functionality, allele frequency, and diplotype to phenotype mapping to go along with each CPIC guideline. You can also obtain these gene-specific information tables from PharmGKB.(23) Moreover, the Pharmacogenomics Clinical Annotation Tool is provided to facilitate the comprehension and reporting of pharmacogenomic-based dose recommendations, such as *SLCO1B1* recommendations.(16)

Individuals are categorized into the following CPIC-recommended phenotype groups for *SLCO1B1* genotype-based phenotypic categorization: poor, decreased, possibly decreased, normal, and increased function phenotypes. PharmGKB and CPIC provide the Diplotype-Phenotype Table, which lists every conceivable genotype (diplotype) of *SLCO1B1* together with the phenotypes that correspond with it.(23) Additionally, templates for reporting the genotype and phenotype frequencies of the *SLCO1B1* allele can be found on the PharmVar *SLCO1B1* gene page under "Other Documents" and under "Publication Tips" on PharmGKB. Structured data makes reporting more transparent and makes the content accessible to PharmGKB and other organizations for further curation.(16)

More and more statins, especially simvastatin, are chosen and dosed according to *SLCO1B1* genetic information. Genetic differences in the gene encoding the drug metabolizing enzyme CYP2C9 and in another transporter, ABCG2, have been shown to be significant for the prescription of statins, in

addition to the *SLCO1B1* genotype. According to the most recent statin guidelines from the CPIC(3), people with weak or diminished *SLCO1B1* function had less transport into the hepatocytes. Therefore, as was already indicated, a range of SAMSs may arise from increased systemic exposure of statins to skeletal muscle tissue. For patients with diminished or impaired *SLCO1B1* function, a lower starting dose of a statin medicine (e.g. rosuvastatin, pitavastatin, atorvastatin, fluvastatin, pravastatin) or an alternate statin (e.g., simvastatin and lovastatin) is indicated.(3) The strongest evidence is for atorvastatin and simvastatin because of their reliance on transporter-mediated distribution. Pharmacokinetic-stratified studies on rosuvastatin and pravastatin, however, indicate that systemic statin exposure may still be impacted by the *SLCO1B1* genotype, albeit to a lesser degree. The important CPIC guidelines also take into account combinatorial effects, namely *SLCO1B1* and *CYP2C9* for fluvastatin and *SLCO1B1* and *ABCG2* for rosuvastatin.(3) Collectively, these interactions demonstrate how complex and important it is to fully understand how differences in drug transporters and drug metabolizing genes can impact the therapeutic usage of commonly prescribed cholesterol-lowering drugs.(16)

## 1.7. Croatian Pharmacogenetics

### *1.7.1. Pharmacogenetic Studies*

The majority of people carry SNPs that are important for drug metabolism, according to research. This is also the case in the Republic of Croatia, where it was found that 73.7% of patients had actionable gene-drug pairs at the time of pharmacogenomic testing.(46) Studies on SNPs in the Croatian population started in as early as the beginning of the 21<sup>st</sup> century, when Božina et al. investigated the polymorphisms of *CYP*,(47) and later Ganoci et al. expanded it.(48) These pioneering studies showed that the genetic landscape of Croats is quite similar with the rest of Europe, and helped set

the standard for future studies to come. In later studies however, a bigger focus was put specifically on the Croatian population, so that more personal drug guidelines could be made.

Pharmacogenetic studies in the Croatian population have provided valuable insights into the allele frequencies, genotypes, and phenotypes of drug-metabolizing enzymes, transporters, and receptors, which are crucial for optimizing drug therapy and minimizing ADRs. In one prominent study, 27 loci in 20 absorption, distribution, metabolism, and excretion genes were examined in relation to the European average and the Croatian population. Significant variations were seen in the frequency of certain polymorphisms, with greater frequencies in Croats for *CYP2B6\*4*, *CYP2C9\*2*, and *VKORC1*, and lower frequencies for *GSTP1* and *CYP2A6*. These variations highlight how crucial it is to take population-specific genetic information into account when creating pharmacogenetic guidelines.(49) Another significant research effort involved a comprehensive examination of a 28-gene pharmacogenomic panel in 522 Croatian patients. This study aimed to establish the frequencies of alleles, genotypes, and phenotypes related to drug-metabolizing enzymes and other proteins. The results showed that most allele frequencies were similar to those found in other European populations, although certain genes exhibited higher frequencies of altered metabolism rates. This data is instrumental for developing tailored drug use guidelines to improve therapeutic outcomes and reduce ADRs in the Croatian population.(50) Additionally, specific studies have focused on particular patient groups, such as Croatian breast cancer patients, analyzing polymorphisms like *CYP3A4\*1B*, *CYP3A5\*3*, and *UGT1A4\*2*. These studies revealed that the *CYP3A4\*1B* allele is relatively rare, while the *CYP3A5\*3* polymorphism is prevalent, aligning with findings in other European populations. Understanding these polymorphisms helps in personalizing treatments, such as hormone therapy with anastrozole, and managing side effects more effectively.(51) Overall, these pharmacogenetic studies highlight the distinct genetic makeup of the Croatian population and its

implications for personalized medicine. This research supports the need for population-specific guidelines to enhance drug efficacy and safety. Because doing so may help adapt population-specific actions that may have long-term health and economic effects, it is prudent to consistently record the population-specific frequencies of the most relevant SNPs.

### *1.7.2. Personalised medicine in Croatia*

Personalized medicine in Croatia is an evolving field, leveraging advances in genomics, biotechnology, and data science to tailor medical treatments to individual patients' genetic makeup, lifestyle, and environment. The aim is to improve treatment outcomes and minimize adverse effects by creating more precise interventions.

Croatian universities and research institutions are contributing to the growth of personalized medicine through specialized programs in genomics, bioinformatics, and molecular biology. The University of Zagreb and the Ruđer Bošković Institute play significant roles in advancing research in this area.(42,48,52,53) The Croatian government has recognized the importance of personalized medicine, with support from the Ministry of Health. Croatia's National Health Strategy includes goals for integrating new medical technologies and personalized treatments into the healthcare system. Efforts are underway to develop strategies for the implementation of personalized healthcare in the national healthcare framework, focusing on precision diagnostics and genomics. Hospitals and medical centers, particularly in Zagreb, and lately in Osijek and Rijeka, are beginning to incorporate personalized medicine into their clinical practices, especially in oncology and psychiatry, where genetic testing is used to guide chemotherapy and other treatments. The introduction of molecular profiling in cancer treatment has allowed for more targeted therapies, improving patient outcomes.(54,55) Croatia has also been developing biobanks and genomic databases to store and analyze genetic data. This helps in research

related to population health and personalized medicine applications.(56)  
The future of personalized medicine in Croatia looks promising, with ongoing research and integration of new technologies expected to make treatments more individualized and effective across various medical fields.

## 2. Aim of thesis

The Solute Carrier Organic Anion Transporter Family Member 1B1, encoded by the *SLCO1B1* gene, is a critically important transporter with significant implications for the metabolism and therapeutic efficacy of statins, a class of drugs widely prescribed to lower cholesterol levels and reduce the risk of cardiovascular events. The *SLCO1B1* transporter plays a crucial role in the hepatic uptake of statins from the blood, which in turn influences the pharmacokinetics and pharmacodynamics of these medications. Given its central role in statin therapy, *SLCO1B1* has garnered substantial attention in pharmacogenetic research, as variations in this gene can significantly impact drug response and the incidence of adverse effects, particularly statin-induced myopathy.

As scientific understanding of pharmacogenomics continues to evolve, the relevance of *SLCO1B1* in the individualization and optimization of statin pharmacotherapy is increasingly recognized. Among the various polymorphisms identified within the *SLCO1B1* gene, the c.521T>C polymorphism has been the most extensively studied. This variant is associated with reduced transporter function, leading to higher systemic concentrations of statins, which can increase the risk of side effects such as muscle toxicity. Consequently, the c.521T>C polymorphism is well-characterized in populations across the globe, with robust data available on its prevalence and clinical implications.

In contrast, the c.388A>G polymorphism, another variant of the *SLCO1B1* gene, has not been as thoroughly researched. Despite its potential significance in influencing statin pharmacokinetics, there is a relative paucity of data on the frequency and clinical impact of the c.388A>G polymorphism, particularly in specific populations such as those in Croatia. Understanding the distribution of this polymorphism, along with the c.521T>C variant, in the Croatian population is crucial for the development

of tailored therapeutic strategies that maximize efficacy and minimize adverse effects.

To address this gap in knowledge, genotyping of the *SLCO1B1* gene is routinely conducted at the Department of Pharmacogenomics and Individualization of Therapy within the Clinical Institute for Laboratory Diagnostics at the Clinical Hospital Center Zagreb. This initiative aims to generate comprehensive data on the prevalence of the c.521T>C and c.388A>G polymorphisms in the Croatian population, thereby contributing to the global understanding of these variants.

The primary objective of this study is to determine the frequencies of the c.521T>C and c.388A>G polymorphisms in a representative sample of the Croatian population. Additionally, it seeks to compare these frequencies with those observed in other European populations, thereby providing insights into potential genetic similarities or differences.

Beyond frequency analysis, this study also explores potential correlations between these polymorphisms and various demographic variables, including gender, age, and year of birth. This analysis aims to identify any significant associations that could inform personalized treatment approaches. Moreover, it underscores the importance of pharmacogenetic testing as a valuable tool in clinical practice, facilitating the personalization of statin therapy to achieve optimal therapeutic outcomes while minimizing the risk of adverse effects.

The findings of this research have broader implications for public health and clinical practice in Croatia. By demonstrating the utility of pharmacogenetic analysis in guiding statin therapy, this study advocates for the integration of genetic testing into routine clinical care. Such an approach could enhance the efficacy of statin treatment, reduce the incidence of drug-related complications, and ultimately lead to more personalized and effective healthcare. This study also highlights the potential for pharmacogenetic testing to inform health policy, encouraging government and healthcare

institutions in Croatia to consider the implementation of gene testing as part of a comprehensive strategy to improve patient outcomes in statin therapy.



### **3. Subjects and methods**

#### 3.1. Subjects

The study comprised a total of 459 anonymized participants of Caucasian European ancestry who were all citizens of Croatia, including 217 females and 242 males (median age being 58 years; range being 3-88 years). Over an eight-year period (2009-2016), data was gathered from the individuals who underwent genotyping at the University Hospital Center Zagreb.

#### 3.2. Genotyping

Samples of peripheral/whole blood were collected from the participants. Next, in order to perform genotyping, genomic DNA was isolated from it using the QIAamp® DNA Blood Mini Kit (50) (Qiagen, Hilden, Germany) (Figure 5). The kit contains the following: QIAamp Mini Spin Columns, Collection Tubes (2 ml), Buffer AL (lysis buffer), Buffer AW1 (concentrate) (wash buffer 1), Buffer AW2 (concentrate) (wash buffer 2), Buffer AE (elution buffer), QIAGEN® Protease and Protease Solvent.

##### *3.2.1. Extraction of DNA from peripheral/whole blood using the QIAamp method*

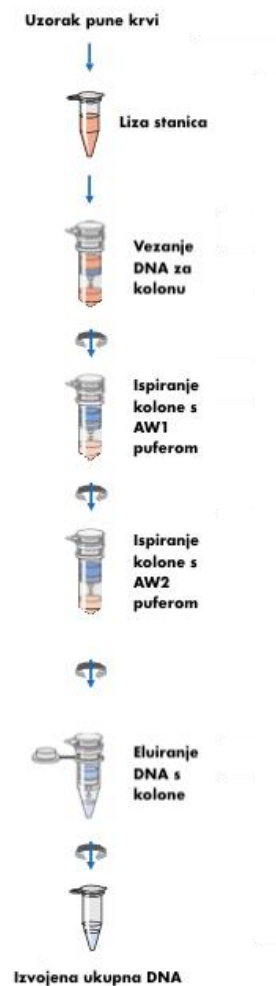
Before extraction, buffers and reagents were prepared. This includes pipetting 1.2 ml of the protease solvent into the vial containing the lyophilized QIAGEN Protease when using the QIAamp DNA Blood Mini Kit (50), as directed on the label. Another crucial step is adding 96-100% ethanol (Sigma-Aldrich, St. Louis, MO, USA) to the AW1 and AW2 concentrate bottles before usage, in accordance with the instructions. The Buffer AL was also well shaken before proceeding. In addition, samples and Buffer AE were brought to room temperature, and a heating block

(Eppendorf ThermoStat C, Eppendorf, Hamburg, Germany) was heated to 56°C.

Once prepared, 20 µL of QIAGEN Protease were pipetted into a 1.5 mL microcentrifuge tube, after which 200 µL of whole blood sample and 200 µL of Buffer AL were added. The microcentrifuge tube was then shaken on a vortex mixer (V-1 plus, BioSan, Riga, Latvia) for 15 s before being incubated for 10 minutes at 56 °C (Eppendorf ThermoStat C, Eppendorf, Hamburg, Germany). A centrifuge of the microcentrifuge tube was conducted briefly in a microcentrifuge (Eppendorf Centrifuge 5424, Eppendorf, Hamburg, Germany) to lower the droplets from the inside of the lid. After that, 200 µL of 96-100% ethanol were added and once again the microcentrifuge tube was briefly shaken on a vortex mixer then centrifuged as described earlier. This was done for each patient's whole blood sample.

Next, the QIAamp Mini Spin Columns were prepared and placed in collection tubes of 2 mL. The mixtures of patient's samples gained in the previous section were added to the columns without wetting the rim before closing, and centrifuged for 1 minute at 6000 g (8000 rpm). Once the centrifuge was completed, the collection tubes with the filtrate were thrown away, and the columns were placed in new clean 2 ml collection tubes. The columns were then carefully opened in order to add 500 µL of Buffer AW1 while paying attention as to not wet the lid. The columns were then closed and centrifuged once more for 1 minute at 6000 g (8000 rpm). After this, the full collection tubes with the filtrate were thrown away with the columns being placed in new, clean 2 mL collection tubes. Like before, the columns were then carefully opened in order to add 500 µL of Buffer AW2 while paying attention to the lid. The columns were then closed, and this time centrifuged for 3 minutes at the maximum speed, that being 20,000 g (14,000 rpm). The full collection tubes with the filtrate were then, once again, thrown away, and the columns were placed in new, clean 2 mL collection tubes, only to be closed and centrifuged a second time, for 1 minute this time, at the maximum speed of 20,000 g (14,000 rpm). The

collection tubes with the filtrate were then thrown away one last time, with the columns being placed in clean 1.5 microcentrifuge tubes. To these, 200  $\mu$ L of Buffer AE were added and the columns were then incubated for 1 minute at room temperature (15-25 °C). Finally, the columns were closed and centrifuged one last time for 1 minute at 6000 g (8000 rpm). After this, the columns were discarded, and the eluate microcentrifuge tubes containing the isolated DNA in Buffer AE were stored at -15 to -30°C, awaiting PCR amplification.



**Figure 5.** DNA extraction procedure on QIAamp® centrifugation columns. Adapted from QIAGEN, 2016(57)

### *3.2.2. Determination of concentrations and purity of extracted DNA*

To determine the concentration and purity of DNA, a NanoDrop 2000 spectrophotometer (ThermoFischer Scientific, Waltham, MA, USA) was used. Before that, the spectrophotometer had to be calibrated. This was done by pipetting 1.5 µL of Buffer TE (Qiagen, Hilden, Germany) onto the device and running it. The buffer droplet was then wiped with cellulose tissue and the same volume of the isolated DNA in Buffer AE from the previous section was pipetted. Before pipetting however, the sample had to be homogenized using a vortex mixer. The device measured the concentration and purity of each sample. This was done for every sample whilst making sure to wipe the drop of the tested sample between each reading.

The spectrophotometer measured and calculated DNA concentration based on the optical density (OD) of the samples at a wavelength of 260 nm. The formula used to calculate the concentration of DNA was:

$$\text{Concentration of DNA } (\mu\text{g/mL}) = \text{OD}_{260} \times 50 \mu\text{g/mL}$$

The purity of DNA was determined by measuring the OD at a wavelength of 260 ( $A_{260}$ ) and 280 ( $A_{280}$ ) nm.  $A_{260}/A_{280}$  reading ratios of 1.7-1.9 indicated high purity of protein-free DNA in the samples.

### *3.2.3. Genotyping of SLCO1B1 polymorphisms using the TaqMan<sup>®</sup> method*

After extracting DNA samples, PCR was needed for the amplification of said samples. The 7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), specifically with the TaqMan Drug Metabolism Genotyping Assay (TaqMan<sup>®</sup> DME Genotyping Assays, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), were used to achieve this. It's important to note that all genotyping techniques utilized in this research were used in normal diagnostic procedures, and they are

all subject to external quality assurance (EQA) programs or method validation.

As before, preparation of DNA samples, a Reaction mix and the Reaction plate is crucial. The samples were the first to be prepared. Because the sample volume in the 7500 software is restricted to 10% of the entire reaction volume, sample dilutions are required. Dilutions of the separated DNA also needed to be made in a ratio of 1:10 so that the final amount of DNA in the reaction mixture is 1-20ng. These were done in 8-Tube Strips of 0.2 mL placed on the plate rack for PCR. After labeling the microtubes, 30  $\mu$ L of sterile distilled water for PCR and then 3  $\mu$ L of the appropriate DNA solution were first pipetted into each. Before pipetting however, each DNA sample had to have been mixed for 3-5 seconds on a vortex mixer.

The 7500 software also determines which Reaction mix components to use based on the selections made in the Methods and Materials screen. Before it can be made, the components need to be prepared. Namely, the TaqMan<sup>®</sup> DME Genotyping Assay mix needs to be briefly centrifuged on a microcentrifuge (MiniSpin, Eppendorf, Hamburg, Germany) after being temporarily resuspended by vortexing and the TaqMan<sup>®</sup> Universal PCR Master Mix needs to be well mixed by swirling the bottle. With this and the software calculations in mind, a Reaction mix was made in 2 separate sterile 1.5 mL microtubes in order to obtain reaction mixtures for PCR for the two tested polymorphisms (Table 2). First, each component's needed volume was added to each microtube. Then, the Reaction mix was pipetted gently up and down before capping the tube, and briefly centrifuged.

**Table 2.** Ingredients of the Reaction mixture for genotyping *SLCO1B1* by the TaqMan® method

Ingredients of the Reaction mixture for PCR	Volume (µL)*
<i>TaqMan</i> ® Universal PCR Master Mix (1x)	12,5
<i>TaqMan</i> ® DME/SNP Genotyping Assay (1x)	1,25
DNA dilution (1-20 ng)	11,25
<b>Total volume of the reaction mixture:</b>	<b>25 µL</b>

\*The indicated volumes correspond to the reaction mixture for the analysis of one sample. When genotyping multiple samples, the volume of each component must be multiplied by the number of samples being analyzed (including controls). Adapted from *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Genotyping Experiments*, Copyright 2007, 2010 Applied Biosystems, and *TaqMan*® SNP Genotyping Assays User Guide, Copyright 2017 Thermo Fisher Scientific Inc.

Before PCR could be initiated, the 96-Well Reaction plate (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) had to be prepared. This was done by pipetting 13.75 µL of Reaction mixture and 11.25 µL of DNA dilution into each provided well of the microtiter plate. For each tested polymorphism, it was necessary to make a negative and a positive control. Controls were prepared in the same way as tested samples, only 11.25 µL of distilled water was added to the negative control instead of DNA, and 11.25 µL of control DNA of a known genotype to the positive control. One positive control corresponds to the *wt*, and the other to the variant type (*het* for heterozygous or *mut* for mutant). The Reaction plate was then covered with optical adhesive film (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged in a microtiter plate centrifuge (Eppendorf Centrifuge 5810R, Eppendorf, Hamburg, Germany) for 5 seconds at 3000 revolutions per minute. The plate was then placed in the ABI 7500 Real-Time PCR System device and the corresponding program was started. Also, before the actual analysis, it was necessary to name the samples in the wells on the program.

To ensure accurate results, a pre-PCR plate read was performed. The background fluorescence of each well on the plate was captured, which was then later subtracted from the post-read fluorescence during the post-PCR plate read to account for and remove pre-amplification background fluorescence. After this, the Reaction plate was loaded and PCR was conducted using the previously programmed run (Table 3). Once the process was completed, a post-PCR plate read was performed on a real-time PCR instrument.

**Table 3.** PCR reaction conditions on the ABI 7500 device for genotyping

Step	Temperature/°C	Duration	Cycles
Pre-PCR read	60	1 min	HOLD
Polymerase Activation	50, 95	2 min, 10 min	HOLD
Denaturation	95	15 s	50
Annealing/extension	60	90 s	
Post-PCR read	60	1 min	HOLD

Adapted from *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Genotyping Experiments*, Copyright 2007, 2010 Applied Biosystems, and *TaqMan® SNP Genotyping Assays User Guide*, Copyright 2017 Thermo Fisher Scientific Inc.

After the completion of the PCR, the computer program 7500 Software version 2.3 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) analyzed the detected fluorescent signals by the method of allelic discrimination. Based on the measured fluorescence in each well, the program determined the genotype of each sample and displayed the results in the form of a scatter plot, more precisely an allelic discrimination plot (Figure 6). The x-axis represents the fluorescence intensity of one fluorophore (e.g. VIC®), and the y-axis the fluorescence intensity of another fluorophore (e.g. FAM®), i.e. each axis represents one tested type of allele (wild or variant). Each sample was shown as one point, and all points were

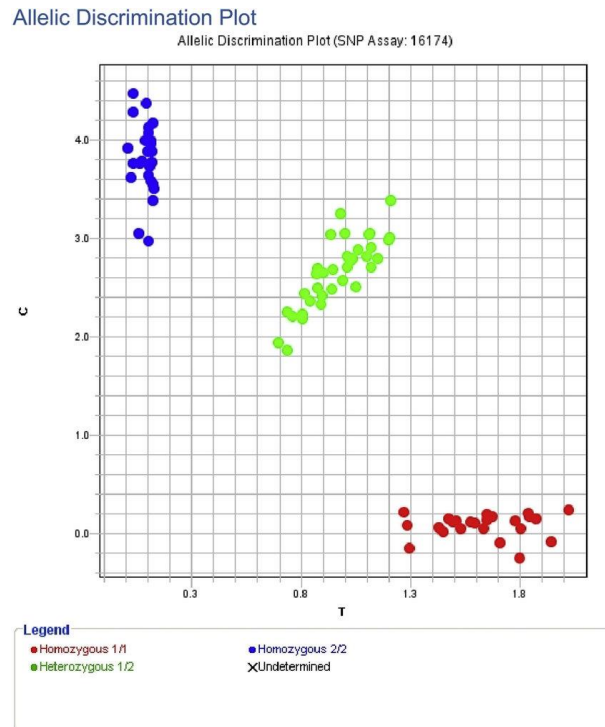
grouped into three separate groups. The groups to which the samples in which only one fluorescent dye was detected (either VIC<sup>®</sup> or FAM<sup>®</sup>) belonged were homozygotes, while the group in which both fluorophores (both VIC<sup>®</sup> and FAM<sup>®</sup>) were detected were heterozygotes. This allowed for the distinction between three genotypes – homozygous of the wild-type allele, heterozygous and homozygous of the variant allele (Table 4). Negative template controls were shown in the diagram as black squares located near the origin.

**Table 4.** Overview of polymorphisms tested by the TaqMan<sup>®</sup> method

Gene	rsSNP*	Nucleotide shift	Nucleotide	Reporter fluorophore
<i>SLCO1B1</i>	<i>rs2306283</i>	c.388A>G	A ( <i>wt</i> )	FAM <sup>®</sup>
			G ( <i>mut</i> )	VIC <sup>®</sup>
<i>SLCO1B1</i>	<i>rs4149056</i>	c.521T>C	T ( <i>wt</i> )	FAM <sup>®</sup>
			C ( <i>mut</i> )	VIC <sup>®</sup>

\* rs# represents the SNP reference number from the dbSNP (Single Nucleotide Polymorphism Database) database





**Figure 6.** Allelic discrimination diagram for the *rs16147* (399 T/C) SNP of NPY.b polymorphism. Red points represent *wt* homozygotes, green points heterozygotes, and blue points *mut* homozygotes. The sign X represents samples of undetermined genotype. Adopted from *Relation of neuropeptide Y gene expression and genotyping with hypertension in chronic kidney disease*, 2019.(58)

### 3.3. Data analysis

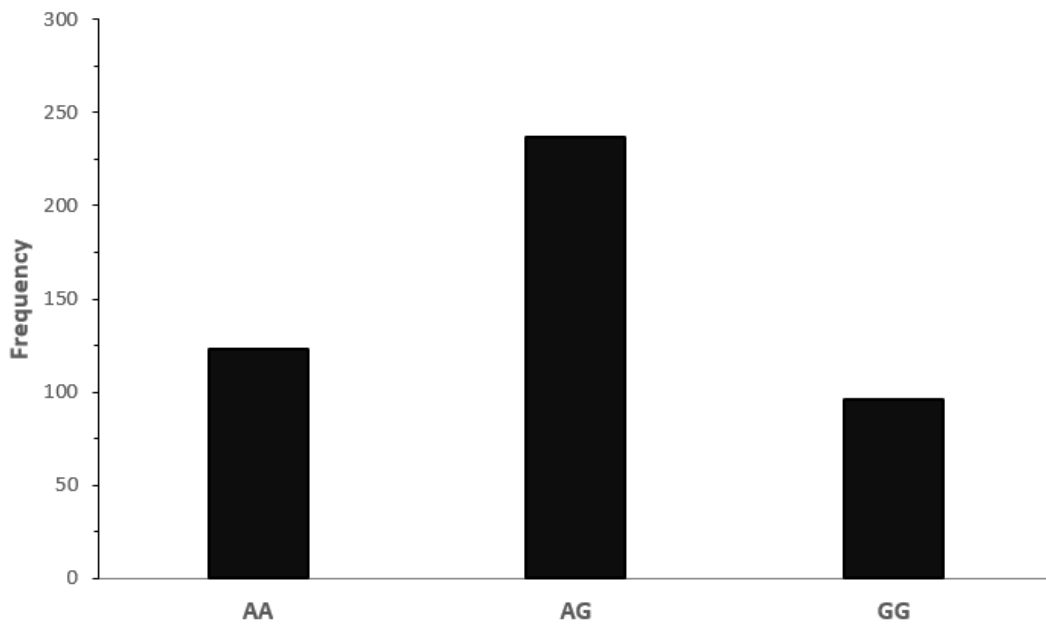
Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) and Statistica (TIBCO Software Inc., Palo Alto, CA, USA) were used for statistical analyses. The gene counting approach was used to estimate the allele and genotype frequencies. The z-test was used to determine statistical significance.

## 4. Results

A group of subjects was collected over 8 years in the population of Croatia, in order to determine the allele frequency of the most important polymorphisms of the *SLCO1B1* gene: the *SLCO1B1* c.388A>G and the *SLCO1B1* c.521T>C. The group consisted of a total of 459 respondents: 217 females and 242 males, aged from 3 to 88 years (median 58 years). For 3 of the 459 respondents, the data regarding the polymorphism of the *SLCO1B1* c.388A>G was not collected.

### 4.1. Allele frequency of *SLCO1B1* gene polymorphism c.388A>G

Out of 456 subjects, more than half (51.97%) were heterozygous (genotype 388A/G) for the *SLCO1B1* c.388A>G gene (Figure 7). The second biggest group (26.97%) were the subjects with the homozygous *wt* allele (genotype 388A/A), while the remaining 21.05% of subjects were homozygous of the variant allele (genotype 388G/G). Unsurprisingly, the frequencies of the alleles were almost split evenly (Table 5), with the *wt* allele (A) frequency having a slight edge (52.96%) over the the frequency of the variant (*mut*) (47.04%).



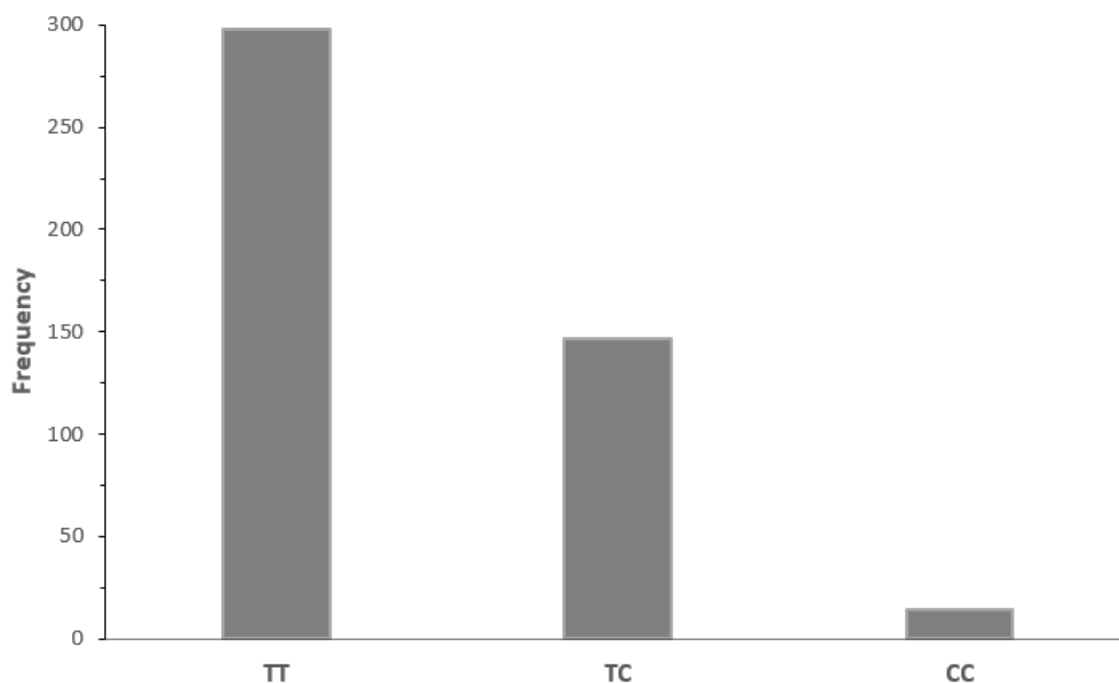
**Figure 7. Frequency and genotype distribution of the *SLCO1B1* c.388A>G polymorphism.** DNA of 456 participants was isolated from blood samples and amplified using the TaqMan<sup>®</sup> method. Data are presented as counted frequencies.

**Table 5. *SLCO1B1* c.388A>G total number of A and G alleles among genotypes**

	genotype 388A/A	genotype 388A/G	genotype 388G/G	Total allele number
# of participants, N	123	237	96	/
# of A alleles	246	237	0	483
# of G alleles	0	237	192	429

#### 4.2. Allele frequency of *SLCO1B1* gene polymorphism c.521T>C

Out of 459 subjects, almost two thirds of them (64.92%) were homozygous *wt* allele for *SLCO1B1* c.521T>C (genotype 521T/T) (Figure 8). Another third (32.03%) belonged to the ones who were heterozygous (genotype 521T/C). The remaining minority (3.05%) was comprised of solely homozygotes of the variant allele (genotype 521C/C). Following this, an overwhelming majority of the frequencies of the alleles (80.94%) belonged to the frequency of the *wt* allele (T), while the rest (19.06%) belonged to the frequency of the variant (*mut*) allele (C) (Table 6).



**Figure 8. Frequency and genotype distribution of the *SLCO1B1* c.521T>C polymorphism.** DNA of 459 participants was isolated from blood samples and amplified using the TaqMan® method. Data are presented as counted frequencies.

**Table 6.** *SLCO1B1* c.521T>C total number of T and C alleles among genotypes

	genotype 521T/T	genotype 521T/C	genotype 521C/C	Total allele number
# of participants, N	298	147	14	/
# of T alleles	596	147	0	743
# of C alleles	0	147	28	175

#### 4.3. Effects of certain variables on allele frequencies of *SLCO1B1* gene polymorphisms c.388A>G and c.521T>C

In this study, the divide between the genders of the participants is almost equal, with around 53% of them being male, and 47% being female (Table 7). A vast majority of them are 50 years old or older, while only a few are considered as minors. However, when it comes to the year of birth, the participants are, more or less, split evenly between 1920s-1950s and 1950s-2010s (Table 8).

**Table 7.** Distribution of age between genders.

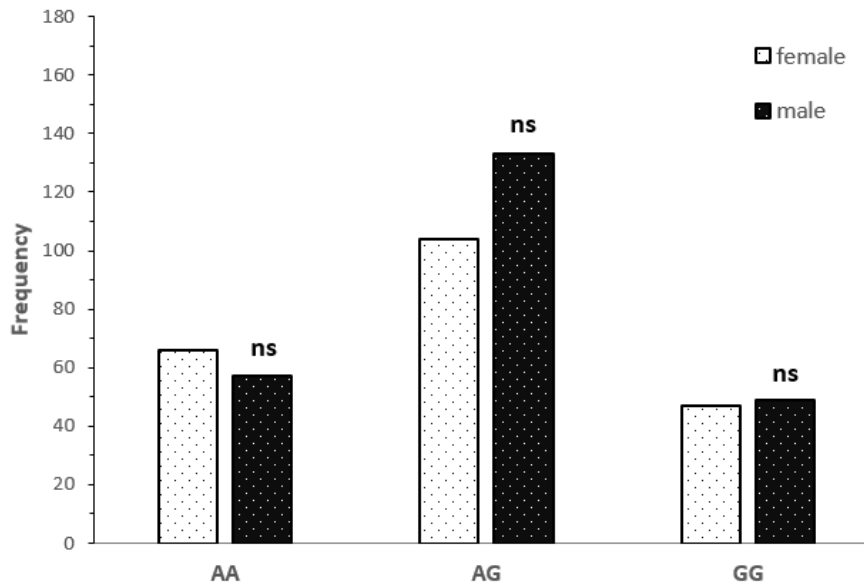
Gender / Age range	0-17	18-49	50+	Total
Male	5	79	158	242
Female	3	35	179	217
<b>Total</b>	<b>8</b>	<b>114</b>	<b>337</b>	<b>459</b>

**Table 8.** Distribution of year of birth between genders.

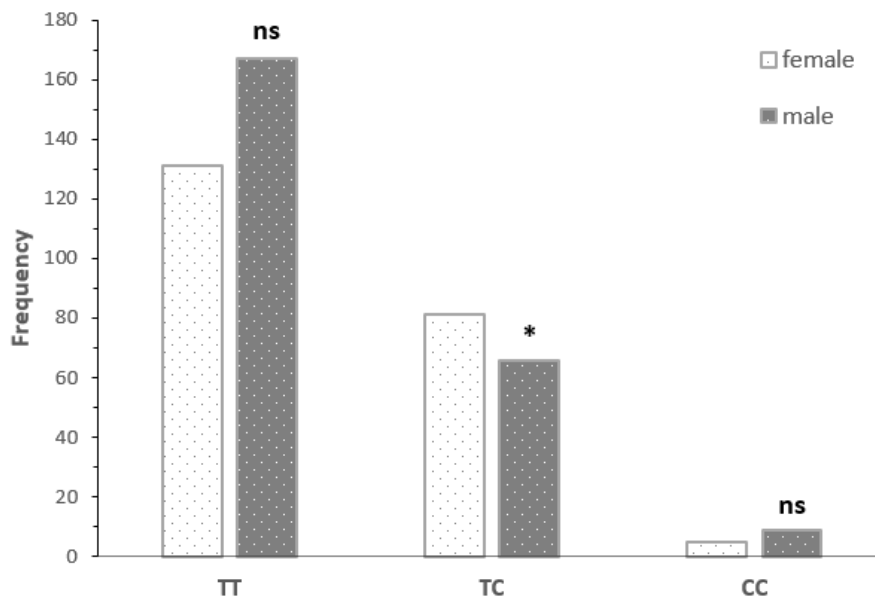
Gender / YoB range	1924-1953	1954-2010	Total
Male	101	141	242
Female	132	85	217
<b>Total</b>	<b>233</b>	<b>226</b>	<b>459</b>

#### 4.3.1. Gender

When it came to the *SLCO1B1* c.388A>G polymorphism, there wasn't any statistical significance in its distribution between the two genders. In fact, for all three genotypes, the ratios were almost 50:50 (Figure 9). However, the same couldn't be said for the *SLCO1B1* c.521T>C polymorphism (Figure 10). Namely, there was statistical correlation between gender and the heterozygous genotype (521T/C). The same could have almost been said for gender and the homozygous *wt* genotype (521T/T) ( $p = 0.0528$ ). No other correlations were found.



**Figure 9. Gender has no statistically significant impact on the *SLCO1B1* c.388A>G polymorphism distribution.** DNA of 456 participants was isolated from blood samples and amplified using the TaqMan® method. Data are presented as counted frequencies. ns: not significant ( $p > 0.05$ ) for female vs male, z-test,  $n =$  counted frequency.

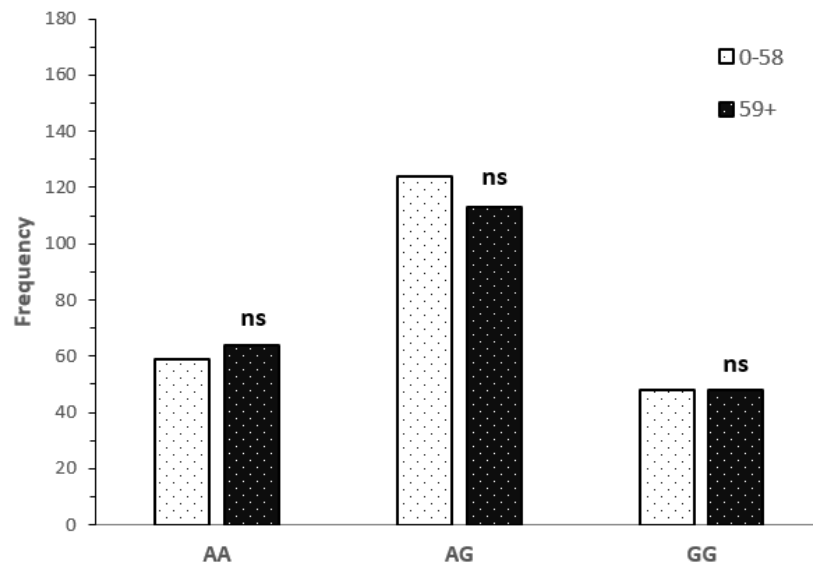


**Figure 10. Gender may have a statistically significant impact on the *SLCO1B1* c.521T>C polymorphism distribution.** DNA of 459 participants was isolated from blood samples and amplified using the TaqMan® method. Data

are presented as counted frequencies. ns: not significant ( $p > 0.05$ ), \*:  $p < 0.05$  for female vs male, z-test,  $n =$  counted frequency.

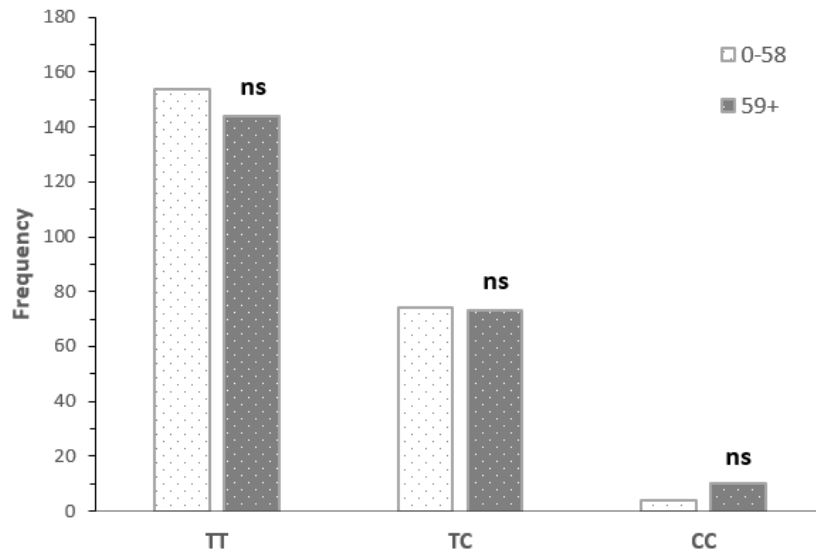
#### 4.3.2. Age

Unlike with gender, there didn't seem to be any statistically significant correlation between the participant's age and their genotype, for both the *SLCO1B1* c.388A>G (Figure 11) and the *SLCO1B1* c.521T>C polymorphisms (Figure 12). In fact, for all of the genotypes, the ratios were almost equally split.



**Figure 11. Age has no statistically significant impact on the *SLCO1B1* c.388A>G polymorphism distribution.** DNA of 456 participants was isolated from blood samples and amplified using the TaqMan® method. Data are presented as counted frequencies. ns: not significant ( $p > 0.05$ ) for female vs male, z-test,  $n =$  counted frequency.

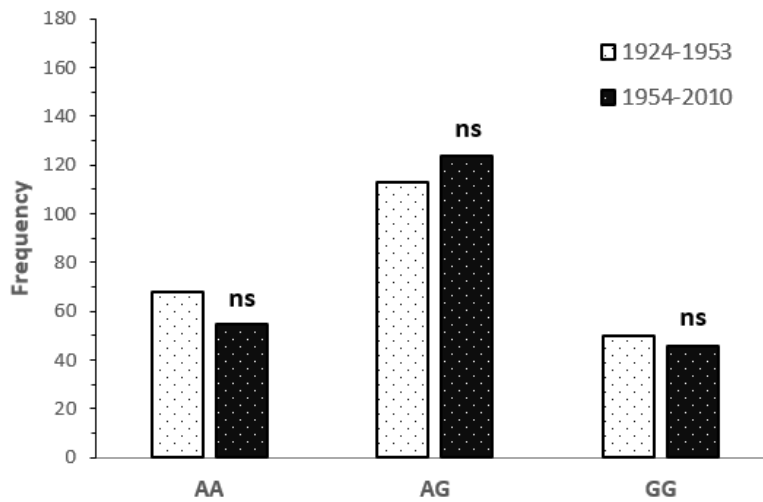




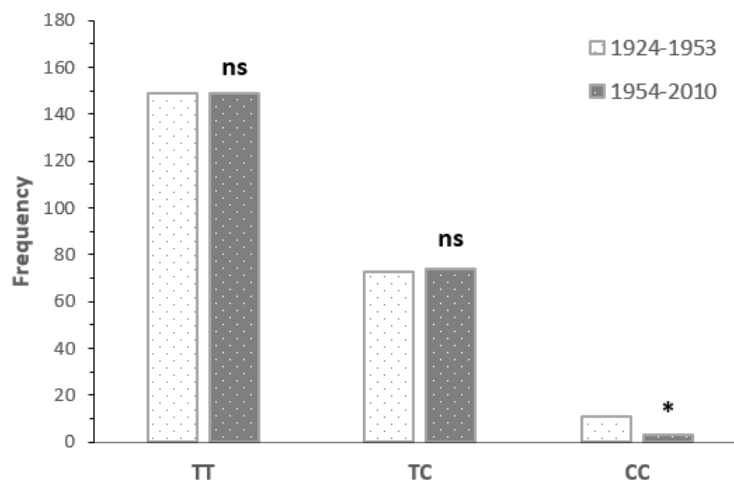
**Figure 12. Age has no statistically significant impact on the *SLCO1B1* c.521T>C polymorphism distribution.** DNA of 459 participants was isolated from blood samples and amplified using the TaqMan® method. Data are presented as counted frequencies. ns: not significant ( $p > 0.05$ ) for female vs male, z-test,  $n =$  counted frequency.

#### 4.3.3. Year of birth

Lastly, upon analyzing the correlations between the year of birth of participants and the polymorphisms, there wasn't any statistical significance when it came to the *SLCO1B1* c.388A>G SNP. Once again, for all three genotypes, the ratios were mostly split down the middle (Figure 13). However, the same didn't apply to the *SLCO1B1* c.521T>C polymorphism (Figure 14). Namely, there was statistical correlation between the homozygous *mut* genotype (521C/C) and the year of birth. No other correlations were found.



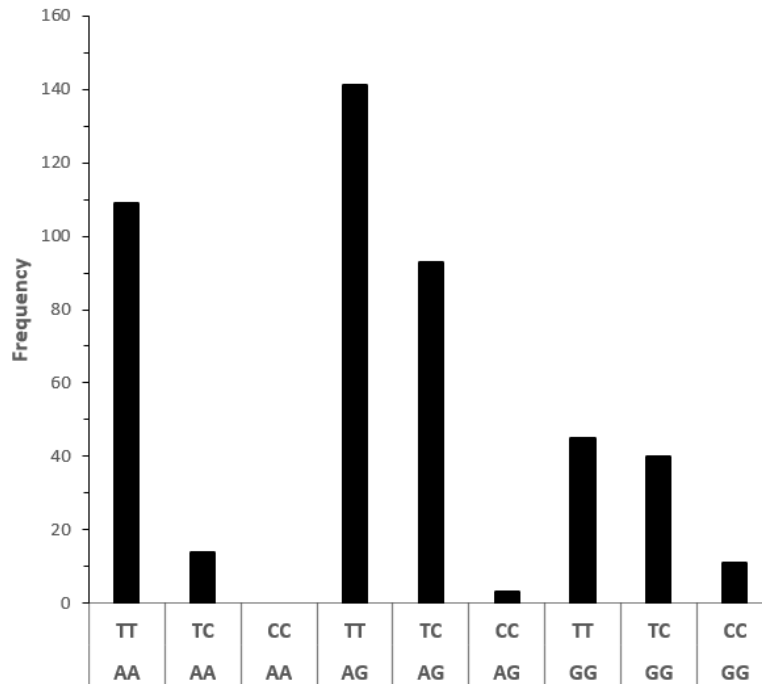
**Figure 13. Year of birth has no statistically significant impact on the *SLCO1B1* c.388A>G polymorphism distribution.** DNA of 456 participants was isolated from blood samples and amplified using the TaqMan® method. Data are presented as counted frequencies. ns: not significant ( $p > 0.05$ ) for female vs male, z-test,  $n =$  counted frequency.



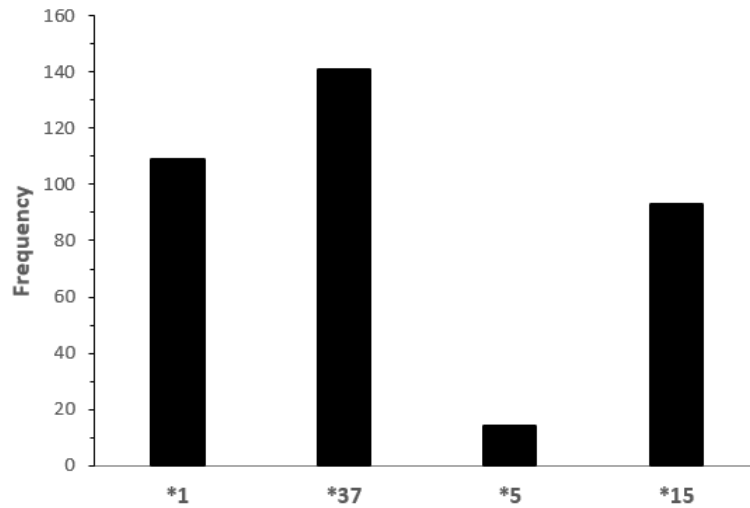
**Figure 14. Year of birth may have a statistically significant impact on the *SLCO1B1* c.521T>C polymorphism distribution.** DNA of 459 participants was isolated from blood samples and amplified using the TaqMan® method. Data are presented as counted frequencies. ns: not significant ( $p > 0.05$ ), \*:  $p < 0.05$  for female vs male, z-test,  $n =$  counted frequency.

#### 4.4. Combination of *SLCO1B1* genotypes c.388A>G and c.521T>C among subjects

A total of 9 different combinations of polymorphisms could be observed in this research (Figure 15). The most common one (30.92%) was the pairing of the heterozygous 388A/G genotype of *SLCO1B1* c.388A>G with the homozygous *wt* 521T/T genotype of the c.521T>C polymorphism. Following closely behind were the combo of both *wt* polymorphisms (388A/A & 521T/T) and the combo of both heterozygous polymorphisms (388A/G & 521T/C) with 23.90% and 20.39% respectively. Other combinations were much less prevalent, while the homozygous *wt* 388A/A with the homozygous *mut* 521C/C polymorphism combo had no prevalence. This means that, when looking at the haplotypes, the \*37 was the most frequent (30.72%), followed by \*1 (23.75%) and \*15 (20.26%), with the \*5 being the rarest (3.05%) (Figure 16).



**Figure 15. Frequency of *SLCO1B1* c.388A>G and *SLCO1B1* c.521T>C polymorphism combinations.** DNA of 459 participants was isolated from blood samples and amplified using the TaqMan® method. Data are presented as counted frequencies. Top row are c.521T>C genotypes while bottom row are c.388A>G genotypes.



**Figure 16. Frequency of *SLCO1B1* haplotypes.** DNA of 459 participants was isolated from blood samples and amplified using the TaqMan® method. Data are presented as counted frequencies. \*1 haplotype contains both *wt* genotypes, \*37 contains the c.388A>G polymorphism, \*5 contains the c.521T>C polymorphism and the \*15 contains both heterozygous genotypes.

## 5. Discussion

The results of this current study align with other studies analyzing *SLCO1B1* variant distribution in Croatia (Table 9). This study analyzed the frequencies of two key *SLCO1B1* polymorphisms, c.388A>G and c.521T>C, within the Croatian population. Out of the 456 subjects, a little over half were found to be heterozygous (388A/G) for the *SLCO1B1* c.388A>G polymorphism. The next largest group, comprising 26.97% of the subjects, were homozygous for the wild-type allele (388A/A), while 21.05% were homozygous for the variant allele (388G/G). The allele frequencies were almost evenly distributed, with the wild-type allele (A) having a slight majority at 52.96% compared to the variant allele (G) at 47.04%. These results are similar to the findings of a study done by Mirošević Skvrce et al., where, out of 150 participants, 44.67% had the AG genotype, a little over a third had the AA genotype, and the remaining fifth had the GG genotype. The allele frequencies were also almost evenly distributed (59% wild-type A vs. 41% variant G).<sup>(52)</sup> Next, for the *SLCO1B1* c.521T>C polymorphism, in the current study nearly two-thirds were homozygous for the wild-type allele (521T/T) of the *SLCO1B1* c.521T>C polymorphism. About one-third were heterozygous (521T/C), while the remaining small minority consisted of individuals who were homozygous for the variant allele (521C/C). This means the wild-type allele (T) was predominant, with a frequency of 80.94%, while the variant allele (C) was found at 19.06%. These results are very comparable to a study done by Božina et al., where the results of 905 subjects were as follows: 61.7% were of the TT genotype, 34.8% of the TC genotype and 3.5% of the CC genotype. The frequency of the C allele was 20.9%. This study didn't analyze the c.388A>G variant though.<sup>(53)</sup> Moreover, this current study's results are also comparable to the Mirošević Skvrce et al. study, where the frequency of TT, TC and CC genotypes was 68.67%, 28.67% and 2.67% respectively.<sup>(52)</sup> Finally, in a research paper by Celinščak et al., they found that the variant C allele frequency of the Croatian population was 0.1735 or 17.35% by pooling their data and of two

other studies.(49) These findings emphasize the importance of considering genetic polymorphisms in clinical settings to optimize drug efficacy and safety, tailoring medical treatments to the genetic profiles of patients.

**Table 9.** Comparison of genotype and allele frequencies across studies.

Genotype/Allele	This study	Božina et al.(53)	Mirošević Skvrce et al.(52)
388A/A	123 ( <b>26.97%</b> )	-	55 ( <b>36.67%</b> )
388A/G	237 ( <b>51.97%</b> )	-	67 ( <b>44.67%</b> )
388G/G	96 ( <b>21.05%</b> )	-	28 ( <b>18.67%</b> )
# of A alleles	483 ( <b>52.96%</b> )	-	177 ( <b>59.00%</b> )
# of G alleles	429 ( <b>47.04%</b> )	-	123 ( <b>41.00%</b> )
521T/T	298 ( <b>64.92%</b> )	558 ( <b>61.66%</b> )	103 ( <b>68.67%</b> )
521T/C	147 ( <b>32.03%</b> )	315 ( <b>34.81%</b> )	43 ( <b>28.67%</b> )
521C/C	14 ( <b>3.05%</b> )	32 ( <b>3.54%</b> )	4 ( <b>2.67%</b> )
# of T alleles	743 ( <b>80.94%</b> )	<b>79.1%</b>	249 ( <b>83.00%</b> )
# of C alleles	175 ( <b>19.06%</b> )	<b>20.9%</b>	51 ( <b>17.00%</b> )

Data is presented as number of participants (percentage of participants in comparison to all participants of a specific study).

### 5.1. Comparison with European Populations

When compared to other European populations, the frequencies of the *SLCO1B1* polymorphisms in Croatia were found to be similar. Using data from the The 1000 Genomes database,(59) the Božina et al. study found that 69.8% of Europeans had the TT genotype, 28.2% had the TC genotype and the remaining 2% had the CC genotype. The Tuscan population of Italy was even closer to the Croatian one, with the TT, TC and CC genotypes

making 60.7%, 35.5% and 3.7% of the population respectively. Logically, the distributions of alleles were also similar, with the T allele making up 84.4% and the C allele 15.7% of Europeans.(53) On the other hand, the Celinščak et al. study used the gnomAD database and found that 16.06% of Europeans have the C allele.(49) This similarity reinforces the relevance of European pharmacogenetic data in the Croatian context and supports the use of broader European guidelines in local clinical practice.

## 5.2. Gender

This study examined the impact of gender on the distribution of *SLCO1B1* polymorphisms. For the *SLCO1B1* c.388A>G variant, no significant gender differences were observed, suggesting that this polymorphism is equally distributed among men and women in the Croatian population. However, a significant correlation was found between gender and the *SLCO1B1* c.521T>C polymorphism, specifically in the heterozygous genotype (521T/C). This finding suggests potential gender-specific influences on the expression of this polymorphism, warranting further investigation into how these differences might affect drug metabolism and efficacy, particularly in gender-specific treatments.

The relationship between *SLCO1B1* gene variants and gender is a complex and evolving area of research. Although the *SLCO1B1* gene itself is not located on a sex chromosome,(16) and thus its variants are not inherently gender-specific, emerging evidence suggests that gender may influence the expression and impact of these variants on drug metabolism and response. This section explores the nuanced interplay between gender and *SLCO1B1* variants, emphasizing the need for gender-specific considerations in pharmacogenomics.



### 5.2.1. Drug response

Studies have shown that while genetic variants are a major determinant of the expression of the hepatic uptake transporter, encoded by *SLCO1B1*, there is no significant direct association between *SLCO1B1* expression levels and gender.(49,60) This indicates that while genetic variations play a critical role in the function of *SLCO1B1*, gender alone does not significantly influence its expression.

However, when considering drug response and adverse effects, gender differences become more apparent. Some clinical studies have observed that women with certain *SLCO1B1* variants, such as the *rs4149056* polymorphism, experience different drug responses or side effect profiles compared to men. For example, an analysis of drug utilization patterns in an elderly cohort, considering genetic factors including *SLCO1B1*, found significant interactions when analyzing gender disparities.(60) This underscores the importance of including gender as a variable in pharmacogenomic research and clinical practice to better understand and optimize drug therapies.

### 5.2.2. Statin-Induced Myopathy

One area where gender differences are particularly notable is in the context of statin-induced myopathy. Evidence suggests that women may be more susceptible to this adverse effect, and the presence of *SLCO1B1* variants, like *rs4149056*, could exacerbate this risk. This increased susceptibility in women may be due to physiological differences such as muscle mass, body fat distribution, and hormone levels, all of which can influence drug metabolism and transport.(61) A study found that women carrying the *SLCO1B1*\*5 genotype were more likely to discontinue statin therapy due to side effects compared to men, further highlighting a gender-specific susceptibility to adverse drug reactions.(62) On the other hand, one study found that males with the AA genotype of the *rs2306283* variant have a

greater response to statin treatment, while those with the GG genotype show somewhat of a resistance.(63) These findings emphasize the need for a gender-specific approach in prescribing and monitoring statin therapy.

### *5.2.3. Hormonal Influence*

Hormones, particularly estrogen, have been shown to modulate the expression of various liver enzymes and transport proteins, including those encoded by the *SLCO1B1* gene. This hormonal modulation can lead to gender differences in the activity of the *SLCO1B1* transporter, potentially altering the impact of *SLCO1B1* variants on drug metabolism.(64) During pregnancy, significant hormonal changes can alter drug metabolism and transport, necessitating adjustments in drug dosing for pregnant women who carry *SLCO1B1* variants. Additionally, a study focusing on menopausal women undergoing hormone therapy found an association between the *SLCO1B1 rs4149056* variant and variations in hormone levels, suggesting that *SLCO1B1* variants might influence the pharmacokinetics of hormone therapy differently in men and women.(64)

While *SLCO1B1* variants themselves are not gender-specific, their effects on drug transport and metabolism can vary between genders due to physiological and hormonal differences. These variations underscore the importance of considering gender in pharmacogenomic research and personalized medicine. By doing so, healthcare providers can optimize drug efficacy and minimize adverse effects for both men and women. Further research is essential to fully understand these gender-specific effects and to incorporate them into clinical guidelines.

### 5.3. Age

Age did not show a significant impact on the distribution of either *SLCO1B1* polymorphism. This result implies that the frequencies of *SLCO1B1*

c.388A>G and c.521T>C are stable across different age groups. Such findings are critical because they allow for the generalization of pharmacogenetic data across diverse populations, ensuring that the genetic information used to predict drug response and tailor therapies is applicable to individuals of all ages. These findings align with other studies that suggest that while genetic variants like *SLCO1B1* c.521T>C significantly influence drug metabolism, age-dependent expression patterns do not appear to significantly alter the distribution of these variants.(65) Nevertheless, a proteomic investigation carried out on liver tissue samples from children revealed age-related variations in the expression of *SLCO1B1* protein among *SLCO1B1* genotype groups.(66) According to another study, compared to adults, children with *SLCO1B1* c.521T>C exhibited a 2-fold greater systemic exposure to simvastatin acid.(67) Still, it's very important to note that not many studies explored the relationship between *SLCO1B1* variants and age in the first place. Evidently, like with other factors discussed here, this scientific topic is relatively fresh, and therefore there is definitely a need for further studies.

#### 5.4. Year of Birth

Interestingly, a significant correlation was observed between the year of birth and the homozygous variant genotype (521C/C) for the *SLCO1B1* c.521T>C polymorphism. This finding could indicate generational shifts in allele frequencies, possibly due to historical changes in population genetics or environmental factors.

While *SLCO1B1* variants are well-documented for their role in influencing clinical outcomes, there is a noticeable gap in the literature when it comes to exploring the relationship between these variants and the year of birth. Current studies tend to investigate the effects of *SLCO1B1* variants across different age groups and populations rather than directly correlating these genetic differences with the year of birth. This could be due to the fact that

year of birth, as a standalone demographic factor, has not been extensively examined in the context of genetic variant expression. Instead, most research has focused on how age and genetic factors together impact pharmacogenomic outcomes, such as drug response and the risk of side effects. While there is evidence that *SLCO1B1* variants influence statin pharmacokinetics and increase the risk of statin-induced side effects like myopathy, this evidence is typically linked to age-related studies. These studies look at how *SLCO1B1* variants affect individuals at different life stages rather than how these variants might vary depending on the specific year of birth. As a result, while *SLCO1B1* variants are known to have significant implications for personalized medicine, there is no clear evidence directly linking these variants to the year of birth.

In summary, while *SLCO1B1* gene variants play a crucial role in drug response and the risk of adverse effects, the relationship between these variants and the year of birth remains underexplored. The existing body of research focuses more broadly on age-related outcomes and the influence of genetic factors across different populations. This suggests a potential area for future research to explore, particularly to see if there are any subtle generational shifts in *SLCO1B1* variant expression that might correlate with year of birth. However, based on current evidence, most studies do not directly correlate *SLCO1B1* variant expression with the year of birth, instead emphasizing broader pharmacogenetic implications.

### 5.5. *SLCO1B1* Genotype Combinations and Statins

The study identified a total of nine distinct combinations of the two key *SLCO1B1* polymorphisms—c.388A>G and c.521T>C—among the subjects (Figure 16). These combinations represent various genotypic profiles, each with potential implications for the function of the *SLCO1B1* transporter and, consequently, for the pharmacokinetics of statins in individuals. Among these genotypic combinations, the most prevalent was the heterozygous

388A/G genotype in conjunction with the homozygous wild-type 521T/T genotype, designated as the \*37 haplotype. This combination likely represents a functional variation in the *SLCO1B1* transporter, though the presence of the 388A/G heterozygosity might not influence statin uptake and metabolism as it had been labeled as a normal function haplotype (see PharmGKB allele functionality table(23)). Otherwise, it may increase the transporter function, as indicated by some studies.(21,35) The next most common genotype was the combination of both wild-type polymorphisms, 388A/A and 521T/T, referred to as the \*1 haplotype. Individuals with this genotype are expected to have normal *SLCO1B1* function, as both polymorphisms are in their wild-type form, suggesting that these individuals would typically metabolize statins efficiently with a lower risk of adverse effects such as myopathy. Following this, the combination of both heterozygous polymorphisms, 388A/G and 521T/C, known as the \*15 haplotype, was also relatively common among the study subjects. This genotype suggests a partial impairment of the *SLCO1B1* transporter's function, potentially leading to higher plasma concentrations of statins and an increased risk of side effects compared to those with the wild-type genotypes. The distribution of these genotypic combinations indicates that a little more than half of the Croatian population likely possesses a normal *SLCO1B1* function, characterized by either the \*1 or \*37 haplotypes. This finding implies that these individuals may respond well to standard doses of statins with minimal risk of adverse effects. However, nearly a quarter of the population was found to have genotypes associated with impaired *SLCO1B1* function, namely those carrying the \*5 or \*15 haplotype. These individuals may require dose adjustments or alternative therapies to avoid potential statin-related complications.(39,43)

These findings underscore the genetic diversity within the Croatian population, particularly concerning the *SLCO1B1* gene. The identification of multiple genotypic combinations highlights the complexity of pharmacogenetic interactions and the necessity of considering multiple

polymorphisms when evaluating the pharmacogenetic impact on drug metabolism and efficacy. Moreover, this diversity reinforces the importance of personalized medicine. By recognizing and accounting for these polymorphic variations, healthcare providers can better predict individual responses to statin therapy and tailor treatments to optimize therapeutic outcomes while minimizing the risk of adverse effects. The study's results provide a valuable reference for clinicians and researchers, supporting the ongoing integration of pharmacogenetic testing into routine clinical practice.

#### 5.6. Strengths, Weaknesses and Recommendations for Future Research

This study, like any other, has several strengths. It demonstrated comprehensive genotyping and data analysis by using robust genotyping methods (TaqMan® method) and statistical tools to analyze the frequency of two significant polymorphisms in a large cohort of the Croatian population (459 participants). The detailed methodology for DNA extraction, PCR amplification, and data analysis ensures the reliability and reproducibility of the results. This study also provided valuable population-specific insights into the genetic makeup of the Croatian population, particularly in relation to *SLCO1B1* polymorphisms. This is important for personalized medicine, as it helps tailor drug therapy to the genetic profiles of individuals, potentially improving therapeutic outcomes and reducing adverse drug reactions. Another strength lies in the comparison between allele frequencies of the Croatian population and those of other European populations, which offers a broader context for understanding genetic variability. This comparative approach strengthens the relevance of the findings beyond the Croatian population. Lastly, this study emphasizes the clinical implications of the polymorphisms studied in relation to external factors, such as gender and age. This focus on practical applications in medicine enhances the study's impact and relevance to healthcare.

There are also several weaknesses that plague this study. The first one is limited size (relatively small number of participants) and scope, as the focus is solely on two polymorphisms (c.388A>G and c.521T>C) of the *SLCO1B1* gene. While these are important, the scope is somewhat narrow, as it does not consider other potentially relevant polymorphisms or genes that could influence drug metabolism and efficacy. There is also a lack of functional analysis, as although the study provides data on the frequency of polymorphisms, it lacks data on how these genetic variations affect the function of the *SLCO1B1* transporter or the clinical outcomes in the population studied. Without functional data, the clinical implications of the findings remain somewhat speculative. Finally, there is an issue with a potential sample bias. See, the sample consists of participants from a single country and of European ancestry. This may limit the generalizability of the findings to other ethnic groups or populations, as genetic variability can differ significantly across regions and ethnicities.

Future research should focus on expanding the sample size to validate these findings and explore the functional implications of these polymorphisms in greater detail. Additionally, studies investigating the interaction between *SLCO1B1* variants, and other genetic or environmental factors will provide a more comprehensive understanding of their role in drug metabolism. Studies like Principi et al.(68) have emphasized the need for larger, more detailed studies to confirm and expand upon initial findings.

The results of this study underscore the importance of integrating pharmacogenetic testing into clinical practice in Croatia. Given the significant role of *SLCO1B1* polymorphisms in drug metabolism, particularly for statins, understanding the genetic makeup of the population can enhance drug efficacy and minimize adverse effects. Personalized medicine approaches that consider individual genetic profiles, including *SLCO1B1* variants, are essential for optimizing therapeutic outcomes and reducing the risk of side effects. By continuing to explore the genetic factors influencing drug response, particularly in specific populations like Croatia,

healthcare providers can better tailor treatments to individual patients, ultimately improving healthcare outcomes.



## 6. Conclusion

This study provides a comprehensive analysis of the frequency of the *SLCO1B1* c.388A>G and *SLCO1B1* c.521T>C polymorphisms within the Croatian population, offering valuable insights into the genetic makeup relevant to pharmacogenomics. The findings of this research are significant in the context of personalized medicine, particularly in optimizing statin therapy, where these polymorphisms play a critical role in influencing drug response and the risk of adverse effects such as statin-induced myopathy. The findings also further propagate the need to continue studying gender and age differences for therapy optimization.

The data reveals that the c.388A>G polymorphism is quite prevalent in the Croatian population, with approximately half of the subjects being heterozygous (A/G) and the allele frequencies being nearly evenly split between the wild-type (A) and the variant (G) alleles. On the other hand, the c.521T>C polymorphism shows a much higher frequency of the wild-type allele (T) compared to the variant (C) allele, with a majority of the population being homozygous for the wild-type allele. These findings align well with previous studies conducted in Croatia and other European populations, indicating that the distribution of these polymorphisms is consistent with broader population trends across Europe.

One of the strengths of this study is its focus on a relatively large cohort, which enhances the reliability of the allele frequency estimates and provides a solid foundation for comparing the Croatian population with other European groups. This study also emphasizes the clinical relevance of these polymorphisms, particularly in relation to statin therapy. Given the well-established association between the *SLCO1B1* c.521T>C polymorphism and an increased risk of SIM, the high frequency of the wild-type allele in the Croatian population suggests a lower overall risk for adverse statin reactions, although individuals carrying the variant allele may still be at significant risk.

However, the study is not without its limitations. The analysis is confined to a relatively small number of patients, and only two polymorphisms, which, while important, may not capture the full genetic variability affecting drug response. Additionally, this study does not explore the functional impact of these polymorphisms beyond their frequency, leaving a gap in understanding how these genetic variations may influence clinical outcomes in the Croatian population.

In conclusion, this research contributes to the growing body of knowledge on pharmacogenomics in Croatia, providing critical data that could inform more personalized approaches to statin therapy. By identifying the prevalence of key polymorphisms in the *SLCO1B1* gene, this study lays the groundwork for further research into the functional consequences of these genetic variations and their implications for drug efficacy and safety. The findings also underscore the importance of integrating pharmacogenetic testing into clinical practice, not only to optimize therapeutic outcomes but also to minimize the risk of adverse drug reactions, thereby enhancing patient care in Croatia and potentially other populations with similar genetic profiles.

## 7. Literature

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## 8. Curriculum Vitae (CV)





### Ema Nedeljković

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**Date of birth:** 29/10/1999 | **Nationality:** Croatian | **Gender:** Female | **Phone number:** (+385) 915658626 (Mobile) | **Email address:** [ema.nedeljkovic@student.uniri.hr](mailto:ema.nedeljkovic@student.uniri.hr) | **Email address:** [ema.nedeljkovic0192@gmail.com](mailto:ema.nedeljkovic0192@gmail.com) | **Address:** Bože Vidasa 7, 51000, Rijeka, Croatia (Home)

### ● ABOUT ME

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Dedicated and passionate. Looking to broaden my horizons and conquer new challenges!

### ● WORK EXPERIENCE

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19/07/2022 – 05/10/2022 Rijeka, Croatia

#### **DATA ANALYST STUDENT SERVICE OF UNIVERSITY IN RIJEKA**

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I performed data inspection and import of new and existing students, as well as organized the data in an orderly manner. In addition, I offered help to students that entered the office to the best of my abilities.

26/07/2022 – 08/08/2022 Rijeka, Croatia

#### **JUNIOR ANALYST HIDRO.LAB. D.O.O.**

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I was taught how to perform various analyses of water quality, including but not limited to determinations of: the pH value and conductivity of samples, the mineral and total oil percentage in samples, the oxygen concentration in samples, the calorific value in oil and waste, total oil and fat in water samples, oil concentration using Fourier-Transform Infrared Spectroscopy and gravimetric methods, oxygen and anions percentage in a wastewater sample, and of Chemical Oxygen Demand and Biological Oxygen Demand in water samples.

During that time, I got acquainted with functions of various machines, such as FTIR spectrometer, ICP-MS spectrometer, GC-MS, Ionic Chromatographer, Calorimeter, Spectrophotometer for determination of phosphorus, cyanide and chromates, pH meter, and conductivity meter.

07/06/2021 – 18/06/2021 Rijeka, Croatia

#### **STUDENT INTERNSHIP DEPARTMENT OF PUBLIC HEALTH PGC**

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I worked in the Department for air control, examination of physical factors, living and working environment and biomonitoring. I was taught how to operate a microwave digestion system (specifically the Ethos Easy Advanced Microwave Digestion System), as well as how to make samples for it, and how to process them and prepare them for analysis. I was also walked through and participated in some of the steps of analysing SO<sub>2</sub> i NH<sub>3</sub> air concentrations. In addition, I spent some time in the Analytical department, where I learned more about gas chromatography and spectrophotometry.

### ● EDUCATION AND TRAINING

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03/10/2021 – CURRENT Rijeka, Croatia

#### **GRADUATE COURSE: BIOTECHNOLOGY IN MEDICINE** Department of biotechnology Rijeka

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**Address** Radmile Matejčić 2, 51000, Rijeka, Croatia | **Website** <https://www.biotech.uniri.hr/hr/> |

**Final grade** 4.6 GPA (Scholarship of the city of Rijeka)

30/09/2018 – 19/09/2021 Rijeka, Croatia

#### **UNDERGRADUATE COURSE (BCS): BIOTECHNOLOGY AND DRUG RESEARCH** Department of biotechnology Rijeka

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**Address** Radmile Matejčić 2, 51000, Rijeka, Croatia | **Website** <https://www.biotech.uniri.hr/hr/> |

**Final grade** Magna cum laude, 4.6 GPA (STEM scholarship) | **Thesis** *Fusobacterium nucleatum*: universal colonizer

31/08/2014 – 21/05/2018 Rijeka, Croatia

**SECONDARY EDUCATION** Natural Science and Graphics school of Rijeka

**Address** Vukovarska 58, 51000, Rijeka, Croatia | **Website** <https://www.pgsri.hr/>

### ● **LANGUAGE SKILLS**

Mother tongue(s): **CROATIAN**

Other language(s):

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken production	Spoken interaction	
<b>ENGLISH</b>	C2	C2	C1	C1	C1

*Levels: A1 and A2: Basic user; B1 and B2: Independent user; C1 and C2: Proficient user*

### ● **DIGITAL SKILLS**

Microsoft Word | Microsoft Excel | Microsoft Office | Microsoft Powerpoint | Social Media | Google Drive | Facebook | Instagram | VMD – Visual Molecular Dynamics | AVOGADRO for drawing molecule structure | MarvinSketch | PyMOL + Molecular Visualization Program | UCSF Chimera | SciDAVis | wxMacMolPlt | General Atomic and Molecular Electronic Structure System (GAMESS) | MedCalc Statistical Software | RStudio | Zoom | AutoDock Vina | PropKa | LigPlot+ | Statistical program STATISTICA | NCBI Blast GenBank | Cresset Spark | Outlook | Molecular Docking

### ● **ADDITIONAL TRAINING**

09/12/2021 – CURRENT

**Elective courses**

TOMO Global Health - I participated in several courses about various biological/medical/educational topics.

<https://tomoglobalhealth.wixsite.com/my-site>

### ● **VOLUNTEERING**

23/04/2022 – 23/04/2022 Rijeka

**Impulse festival**

I was in charge of selling tickets and giving passes.

### ● **DRIVING LICENCE**

**Driving Licence:** AM

**Driving Licence:** B