

Picture of transfected HEK293 cells overexpressing *ABCC1* and *SLCO2B1* taken with Revolve, ECHO microscope (San Diego, CA).



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STUDY OF ATORVASTATIN TRANSPORT: FROM FUNDAMENTAL TO TRANSLATIONAL APPROACHES

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SUMMARY

Background

Atorvastatin (ATV) effectively reduces cardiovascular diseases risk by lowering cholesterol levels. However, non-adherence to the treatment ranges from 24% to 56% and is partly due to statin-related myotoxicities (SRM). The risk of developing SRM is dose and concentration-dependent, highlighting the importance of the intracellular ATV pharmacokinetics (PK). At the cellular level, ATV partly crosses the membranes passively but mostly via efflux and influx transporters.

Purpose

This thesis explores the implication of efflux and influx transporters in two complementary axes:

1. A fundamental axis in which investigations aimed at assessing the net impact of overexpressing the influx (*SLCO2B1*) and efflux (*ABCC1*) transporters, as well as the effect of functional single nucleotide polymorphisms (SNP), on ATV transport in HEK293 stable recombinant models.
2. A translational axis in which investigations sought to evaluate the implication of efflux and influx transporters at biological levels of expression as well as the consequences of silencing transporter gene expression on ATV cellular transport in primary skeletal muscle myotubes.

Results

The fundamental investigations confirmed that ATV is a substrate for the efflux transporter ABCC1 and the influx transporter SLCO2B1 in single transfected HEK293 cell models. In double transfectant HEK293 models, we further demonstrated that the increased accumulation caused by OATP2B1 was partially counteracted by the efflux driven by ABCC1. Finally, we associated both SNPs; rs45511401 (c.2012G>T, p.Gly671Val) in *ABCC1* and rs12422149 (c.935G>A p.Arg312Gln) in *SLCO2B1*, with a decrease in ATV intracellular accumulation. We showed that the two SNPs are protective against ATV intracellular

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accumulation, but they exert opposite effects on the activity of their respective protein. The variant in *ABCC1* increases MRP1 activity, while the variant in *SLCO2B1* reduces OATP2B1 activity, thereby increasing the efflux or limiting the entry of the drug from/into the cell, respectively.

In the translational part of this thesis, the experiments were performed on primary cultures of myotubes issued from muscle biopsies of twelve volunteers. The screening of the mRNA expression revealed that, among the efflux and the influx transporters, *ABCC1* and *SLCO2B1* were the most importantly expressed at the transcript level, respectively. Moreover, for every gene assessed, a high inter-individual variability in the mRNA expression levels was observed among the volunteers and the expression of *ABCC1* appeared to correlate with the ATV intracellular accumulation. Repressing the mRNA expression of *ABCC1* and *ABCG2* with siRNAs significantly increased the ATV intracellular accumulation in the differentiated myotubes. Interestingly, the siRNA targeting *ABCG2* had moderate impact on ATV intracellular accumulation in a volunteer expressing the variant rs2231142 (c.421C>A, p.Gln141Lys) in *ABCG2*. Finally, we demonstrated that the reduction in *SLCO1B1* and *SLCO2B1* mRNA expression had a significant but moderate impact on ATV intracellular accumulation only in the volunteers with high expression of efflux transporters.

Conclusion and implications

In both chapters of this thesis, we demonstrated a collaboration between the efflux and influx transporter proteins implicated in ATV cell disposition. Modulating their activity with SNP or their expression with siRNAs had a significant impact on the intracellular accumulation of ATV. If further validated, these observations could contribute to understanding the differential responses and drug interactions with ATV treatment.

RESUME (*French*)

Contexte

L'atorvastatine (ATV) réduit efficacement le risque de maladies cardiovasculaires en abaissant les taux de cholestérol. Cependant, la non-adhérence au traitement varie de 24% à 56% et est en partie due au développement de myotoxicités liées aux statines (SRM). Le risque de développer des SRM dépend de la dose et de la concentration, soulignant l'importance de la pharmacocinétique (PK) intracellulaire de l'ATV. Au niveau cellulaire, l'ATV traverse en partie les membranes passivement mais principalement via des transporteurs d'efflux et d'influx.

Objectifs

Cette thèse explore l'implication des transporteurs d'efflux et d'influx à travers deux axes complémentaires :

- a. Le premier axe concerne des investigations fondamentales qui visent à évaluer l'impact net de la surexpression des transporteurs d'influx (*SLCO2B1*) et d'efflux (*ABCC1*), ainsi que l'effet de polymorphismes génétiques fonctionnels (SNP), sur le transport de l'ATV dans les modèles HEK293 recombinants stables.
- b. Le deuxième axe concerne des investigations plus translationnelles qui visent à évaluer l'implication de ces mêmes transporteurs à des niveaux d'expression biologiques ainsi que l'impact de la répression génique de ces transporteurs sur le transport de l'ATV dans les myotubes musculaires squelettiques primaires.

Résultats

L'investigation fondamentale a confirmé que l'ATV est un substrat du transporteur d'efflux ABCC1 et du transporteur d'influx SLCO2B1 dans des modèles cellulaires HEK293 simplement transfectés. Dans des modèles HEK293 doublement transfectés, nous avons ensuite démontré que l'augmentation de l'accumulation provoquée par SLCO2B1 était partiellement contrecarrée par l'efflux induit par ABCC1. Enfin, nous avons associé deux

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SNPs ; rs45511401 (c.2012G>T, p.Gly671Val) dans *ABCC1* et rs12422149 (c.935G>A, p.Arg312Gln) dans *SLCO2B1*, à une diminution de l'accumulation intracellulaire d'ATV. Nous avons montré que les deux SNP protègent contre l'accumulation intracellulaire d'ATV, mais ils exercent des effets opposés sur l'activité des protéines. Le variant affectant *ABCC1* augmente l'activité de MRP1, tandis que le SNP dans *SLCO2B1* réduit l'activité d'influx d'OATP2B1, augmentant l'efflux ou limitant l'entrée du médicament dans la cellule, respectivement.

Dans la partie translationnelle de cette thèse, les expériences ont été réalisées sur des cultures primaires de myotubes issues de biopsies musculaires de douze volontaires. Le survol de l'expression des ARNm a révélé que, parmi les transporteurs d'efflux et d'influx, *ABCC1* et *SLCO2B1* étaient les plus exprimés, respectivement. De plus, pour chaque gène évalué, une forte variabilité inter-individuelle des niveaux d'expression des ARNm a été observée parmi les volontaires, et l'expression d'*ABCC1* était corrélée à l'accumulation intracellulaire d'ATV. La répression de l'expression des ARNm d'*ABCC1* et *ABCG2* avec des siARN augmentait de manière significative l'accumulation intracellulaire d'ATV dans les myotubes différenciés. De manière intéressante, le siARN ciblant *ABCG2* ont eu un impact modéré sur l'accumulation intracellulaire d'ATV chez un volontaire exprimant le variant rs2231142 (c.421C>A, p.Gln141Lys) dans *ABCG2*. Enfin, nous avons démontré que la réduction de l'expression de l'ARNm de *SLCO1B1* et *SLCO2B1* avait un impact significatif mais modéré sur l'accumulation intracellulaire d'ATV uniquement chez les volontaires présentant des taux élevés de transporteurs d'efflux.

Perspectives et conclusion

Dans les deux chapitres de cette thèse, nous avons démontré une collaboration entre les protéines d'efflux et d'influx impliquées dans le transport de l'ATV. La modulation de leur activité due à un SNP ou de leur expression avec l'utilisation de siARN, a eu un impact significatif sur l'accumulation intracellulaire de l'ATV. Si ces observations sont validées, elles pourraient contribuer à la compréhension des réponses et des interactions médicamenteuses différentielles lors d'un traitement avec l'ATV.

ABBREVIATIONS

ABC:	ATP-binding cassette
ACC:	American College of Cardiology
ADME:	Absorption, distribution, metabolism and excretion
ADP:	Adenosine diphosphate
Ahr:	Aryl hydrocarbon receptor
ATP:	Adenosine triphosphate
ATV:	Atorvastatin
AUC:	Area under the curve
BBB:	Blood-brain-barrier
CAR:	Constitutive Androstane Receptor
CBIP:	Centre belge d'Information Pharmacothérapeutique
CDCA:	Chenodeoxycholic acid
C/D:	Trough concentration/dose
CFDA:	5[6]-carboxyfluorescein diacetate succinimidyl ester
CFTR:	Cystic fibrosis transmembrane conductance regulator
CK:	Creatine kinase
CKD:	Chronic kidney disease
C _{max} :	Maximal concentration
COPD:	Chronic obstructive pulmonary disease
CRP:	C-reactive protein
CVD:	Cardiovascular disease
CYP:	Cytochrome P450
DCA:	Deoxycholic acid
DDD:	Defined Daily Dose
DDI:	Drug-drug interaction
EAS:	European Atherosclerosis Society
EC ₅₀ :	Half maximal effective concentration
EPSP:	European Phenotype Standardization Project

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ESC:	European Society of Cardiology
ESPACOMP:	European Society for Patient Adherence
FDA:	Food and Drug Administration
FEV ₁ :	Expiratory force volume in 1 second
FXR:	Farnesoid X receptor
HDL-C:	High-density lipoprotein
HEK293:	Human embryonic kidney 293
HIV:	Human immunodeficiency virus
HMG-CoA:	3-hydroxy-3-methylglutaryl coenzyme A
HNF:	Hepatocyte nuclear factor
IC ₅₀ :	Half maximal inhibitory concentration
INAMI:	National Institute of Sickness and Disability Insurance
K _m :	Michaelis constant
LDL-C:	Low-density lipoprotein cholesterol
MACS:	Magnetic-activated cell sorting
MAF:	Minor allele frequency
MPAG:	7-O-mycophenolic acid glucuronide
MSD:	Membrane spanning domain
NBD:	Nucleotide binding domain
NLRP3:	NLR family pyrin domain containing 3
NSCLC:	Non-small-cell lung cancer
PCSK9:	Proprotein convertase subtilisin/kexin type 9
PK:	Pharmacokinetic
PM _{2.5} :	Fine particulate matter
PXF:	Pregnane X Factor
RAR:	Retinoic Acid Receptor
RXR:	Retinoid X Receptor
SAM:	Statin-associated myopathy
SCORE2:	Systematic Coronary Risk Estimation (updated)
siRNA:	Small interfering RNA

ABBREVIATIONS

SLC:	Solute carrier protein
SNP:	Single Nucleotide Polymorphism
SRM:	Statin-related myotoxicity
SSRI:	Serotonin reuptake inhibitors
TMD:	Transmembrane domain
ULN:	Upper limit normal
VLDL-C:	Very-low-density lipoprotein cholesterol
$V_{\max}$:	Maximum rate of reaction
WT:	Wild-type

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1. Cardiovascular diseases

A. Epidemiology

Cardiovascular diseases (CVD) are a major public health problem that regroup disorders of the heart and blood vessels including, coronary heart disease, cerebrovascular disease, rheumatic heart disease and other conditions [1]. It is estimated that they cause the death of 17.9 million people each year [1]. In Europe, CVD are responsible of about 45% of the global mortality. In total, it has been estimated that they have caused the death of 4.1 million of European females and males in 2019, according to the report of the European Society of Cardiology (ESC) [2]. The distribution between the sexes is uneven, killing more women (2.2 million) than men (1.9 million) in 2019, representing 46% and 38% of the of deaths respectively [2, 3]. The differences can partly be explained by a differential hormone secretion and cardiac physiology between the genders [4]. The estrogen secretion in women has been identified as a regulator of the cardiovascular system physiology and protective against the oxidative stress, delaying their CVD onset by about 10 years compared to men [5-7]. This is the reason why the CVD incidence in premenopausal women is lower compared to men at the same age which is reversed when comparing postmenopausal women to men at the same age [7]. In postmenopausal women, CVD is even characterized as the major cause of death in rural areas [8].

Although 23% of the premature deaths (before the age of 70) are attributable to CVD, ageing is one of the leading causes influencing their onset in the population over 70 years-old [2, 9]. Advanced age modifies the vascular structure and function of arteries, particularly large arteries, inducing morphological changes of the wall (e.g. thickening, increased vessel diameter) promoting atherosclerosis. The reduction of the vessel lumen will also induce an increase of the blood pressure [9, 10]. Understanding and identifying the modifications that lead to CVD co-morbidities in the elderly population is of major concern knowing that it is estimated that by 2040, more than 275 million of people will be over 60 years-old [11].

When enunciating the disparities in CVD development, mentioning the ethnicities is inevitable. An American study indeed highlighted that African Americans citizens have 2-fold greater odds of stroke than Caucasian Americans. This is partly explained by the

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prevalence of higher blood pressure in the African American population (59%) compared to the Caucasians Americans (47%) or the Asian and Hispanic populations (45% and 44%, respectively) [12, 13]. The socioeconomic context of some countries can amplify the innate ethnicity disparities where racial, health or education remain a brake for those minorities to get the proper medical supervision [14].

The household income, implicating the education, the exposition to particulate matter ($PM_{2.5}$) and the lifestyle habits (e.g. high fat diet, smoke, alcohol), is also highly predictive of CVD development [2, 15]. As an example, the exposition to $PM_{2.5}$ has been associated with ischemic heart disease and stroke mortality [16]. In the European Union in 2019, the average annual weighted $PM_{2.5}/m^3$ was $11.4\mu g/m^3$ [17]. In worldwide high-income countries, the mean $PM_{2.5}$ exposure is $11.9\mu g/m^3$ whereas the average of low-income countries is more than doubled ($25.8\mu g/m^3$) [2]. A meta-analysis of eleven studies, very importantly highlighted that each $10\mu g/m^3$ increase in $PM_{2.5}$ exposition is associated with a 15% raise of the CVD risk [18]. Another indirect influence of the household income on the development of CVD is the propensity of low-income populations to smoke. It has indeed been demonstrated that men, and in a lesser extent, women of this category, have greater odds to smoke. In some countries, the poorest men even have up to 2.5 times than the richest men to smoke. Which in some other countries like Mexico, Mauritania and Georgia was reversed due to social influence [19]. Finally, an unbalanced diet and to a larger extent, an ultra-processed diet rich in trans fatty acids more usually found in lower-income household, have been associated with the development of coronary heart diseases [20].

While CVD seems to have various predisposing factors, elevated levels of low-density lipoprotein (LDL-C), very-low-density lipoprotein (VLDL-C) cholesterol and total cholesterol in the blood are recognized as major contributors to its development [2, 21].

B. Cardiovascular risk event assessment

For primary prevention, as soon as a patient is detected with high cholesterol blood levels, a 10-year fatal CV event risk is estimated with the Systematic Coronary Risk Estimation (SCORE2 or SCORE2-OP) assessment tool based on the age of the patient (Figure 1.1) [22, 23]. For secondary preventions, a statin therapy is initiated whatever the SCORE2

estimation of the patient (Figure 1.2). This methodology has been advised by the ESC/EAS (European Atherosclerosis Society) and is considering the main measurable variables that play a key role in the CV risks event. Those are the age, the gender, the systolic blood pressure, the non-HDLc (mmol/L) and the smoking status. Depending on the European region the patient is issued from, the calculation will be either based on the low, moderate, high or very-high risks chart [22]. The result of the SCORE2 will indicate a low-, moderate, high- or very high-risk for the patient to develop CVD within 10 years.

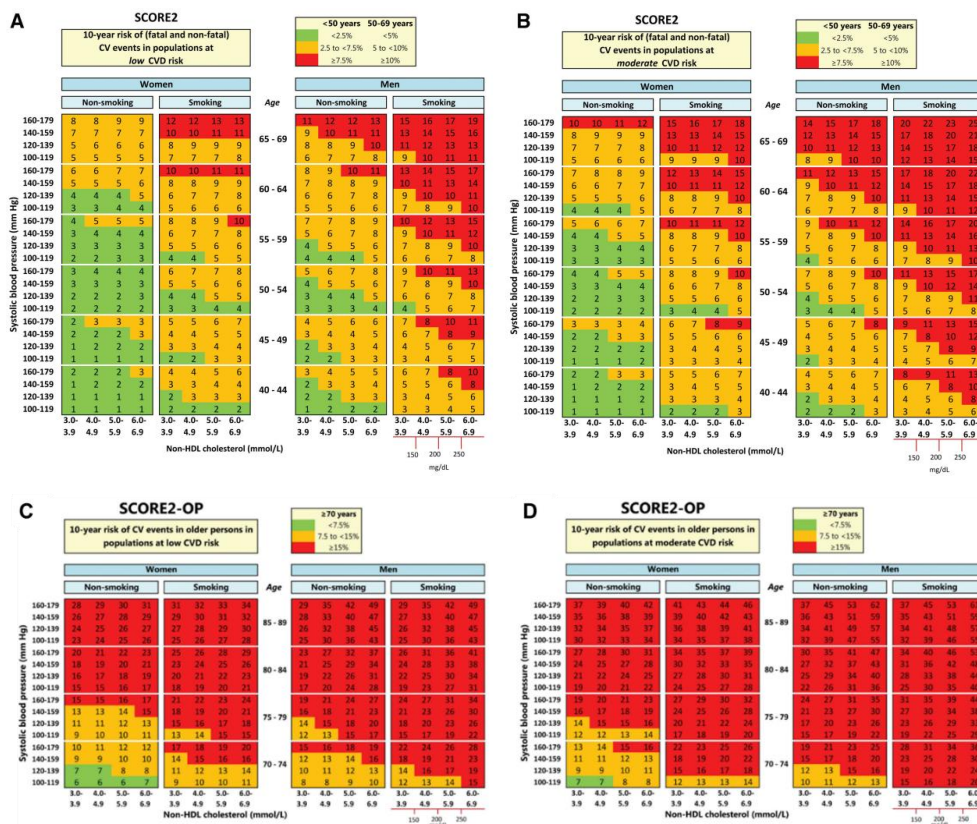


Figure 1.1. Systematic Coronary Risk Estimation (SCORE2 and SCORE2-OP). In the population aged between 40 and 69 years-old in (A) low CVD risk regions of Europe and in (B) moderate CVD risk regions of Europe [22]. In the elderly population aged ≥70 years-old in (C) low CVD risk regions of Europe and in (D) moderate CVD risk regions of Europe [23]. Low risk regions are Norway, Denmark, the UK, the Netherlands, Belgium, Luxembourg, France, Andorra, Monaco, Switzerland, Spain and Israel. Moderate risk regions are Iceland,

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Finland, Sweden, Ireland, Germany, Austria, Italy, Slovenia, Portugal, Cyprus, Malta and Greece. The high and very high CVD risk SCORE2(-OP) tables are not represented and mainly concern the countries from Eastern Europe and Northern Africa. A percentage of 10-years risk of CV event is defined depending on the age, the sex, the systolic blood pressure, the non-HDL-C and the smoking status.

C. Intervention strategy

The SCORE2 is subsequently combined to the LDL-C (mmol/L or mg/dL) to define the intervention strategy (Figure 1.2). The intervention in primary prevention is, for the patients at low risks (highlighted in green on Figure 1.2), giving lifestyle advices. In the moderate cases (identified in yellow on Figure 1.2), a lipid-lowering medication might be considered if an intervention in the lifestyle is not sufficient. In the worst circumstances (pictured in red on Figure 1.2), a lipid-lowering drug is immediately given concomitantly to a lifestyle intervention. As stated above, in secondary prevention, drugs are almost always introduced in the patient's treatment [3, 24]. The decisions should in any case be taken with the patients, which can influence a better adherence to the treatment.

	Total CV risk (SCORE) %	Untreated LDL-C levels					
		<1.4 mmol/L (55 mg/dL)	1.4 to <1.8 mmol/L (55 to <70 mg/dL)	1.8 to <2.6 mmol/L (70 to <100 mg/dL)	2.6 to <3.0 mmol/L (100 to <116 mg/dL)	3.0 to <4.9 mmol/L (116 to <190 mg/dL)	≥4.9 mmol/L (≥190 mg/dL)
Primary prevention	<1, low-risk	Lifestyle advice	Lifestyle advice	Lifestyle advice	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention
	≥1 to <5, or moderate risk (see Table 4)	Lifestyle advice	Lifestyle advice	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention
	≥5 to <10, or high-risk (see Table 4)	Lifestyle advice	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention
	≥10, or at very-high risk due to a risk condition (see Table 4)	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention
Secondary prevention	Very-high-risk	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention

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Figure 1.2. Intervention strategy based on the SCORE2 and the untreated LDL-C levels.
Adapted from the 2019 ESC/EAS Guidelines for the management of dyslipidaemias [3].

D. Place of statins to fight against cardiovascular diseases

3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, have revolutionized the treatment of hypercholesterolemia and the management of patients with increased CVD risk. Decreasing elevated LDL-C levels has indeed proved to be an effective strategy to reduce the risk of CVD in fatal or nonfatal stroke in primary prevention [24, 25]. This has been demonstrated in a meta-analysis performed by Sabatine *et al.* in patients with a mean elevated LDL-C blood levels of 131.5mg/dL. They showed that every 38.7mg/dL LDL-C reduction is associated with a 22% reduction in major CV events [26]. In this study the focus has been set on the LDL-C but controlling the total cholesterol, the triglycerides and the HDL-C are other parameters clinically monitored and targeted to fight

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CVD. The European guidelines for the optimal LDL-C blood rates are; for low-risk patients; <116mg/dL, for moderate-risk patients; <100mg/dL, for high-risk patients; <70mg/dL and in very high-risk patients in primary or secondary prevention; <55mg/dL. The targeted HDL-C levels are between 40 and 60mg/dL, the triglycerides should optimally be <150mg/dL and the total cholesterol <155mg/dL [3]. The risk level is related to Figure 1.1.

To better evaluate the importance of statins utilization across the world, a cross-sectional study aimed at assessing the use of lipid-lowering agents including data from 83 countries (representing 74% of the global population) during the year of 2018. The results showed that 3.1% of the population used lipid-lowering agents and more importantly that 2.6% were under a statin treatment [27]. In Belgium more particularly, the INAMI (National Institute of Sickness and Disability Insurance) reports 1,549,663 statin consumers, which corresponds to 13.5% of insured persons in the country and represents 584,060,902 defined daily dose (DDD) per year [28]. These impressive numbers place statins at the 3rd place of the most prescribed medication delivered by general practitioners in 2021 in Belgium. This indicates the predominance of statins in the treatment of hypercholesterolemia worldwide and in our country. Their supremacy is, in this context, the consequence of their proven high efficiency that influenced the healthcare reimbursement systems to lower their costs. As an example in Belgium, after the intervention of the INAMI, boxes of 100 statins tablets, 10mg or 40mg (e.g. atorvastatin Sandoz) will cost 2.65€ or 6.48€, respectively [29]. The European guidelines indeed recommend the prescription of statins as a first-line therapy to reduce the CV risk event [3]. The recommendations are however flexible in some particular cases. Patients with contraindications (e.g. hepatic impairment, pregnancy) are advised to take another lipid-lowering medication. Those not reaching the controlled cholesterol blood concentrations even at the highest statin tolerated dose, are advised to associate their current treatment to another lipid-lowering method. If patients are suffering from statin-associated muscle side-effect, they are recommended to first switch statin and if no change in the muscle discomfort is observed, other medications will be considered [3, 30]. According to the Centre Belge d'Information Pharmacothérapeutique (CBIP), other lipid-lowering drugs are available on the market. Whereas some are efficient in monotherapy like anion exchange resins (e.g. colestyramine), others are more efficient in combination

with statins. This is the case of fibrates (e.g. fenofibrate, bezafibrate, ciprofibrate), ezetimibe and bempedoic acid. The latter also showed LDL-C reduction effects in combination with ezetimibe. It is to be noted though that those alternatives are also more or less efficient in monotherapy, in case of a severe statin intolerance [29, 31]. The PCSK9 inhibitors (e.g. inclisiran or alirocumab, evolocumab) constitute a drug class that has been demonstrated to significantly reduce high LDL-C levels safely and with great tolerance. However due their relatively new discovery, their efficacy to reduce the mortality and the long term-effects are still unclear [29, 32]. PCSK9 is involved in the degradation of hepatic LDL-receptors, inhibiting this proprotein will then increase LDL-C blood clearance. Alirocumab and evolocumab are antibodies that will directly block its activity while inclisiran is a small interfering RNA repressing the expression of the gene [33, 34]. Some of these alternatives also benefit from clinical care reimbursements, however in a lesser extent than statins. For instance, PCSK9 inhibitors, which are very expensive, are reimbursed by INAMI only if the patient is diagnosed with heterozygous familial hypercholesterolemia [29].

Among the five commercialized statins in the European Union, that are atorvastatin (ATV), simvastatin, rosuvastatin, pravastatin and fluvastatin, ATV stands out as being the most prescribed in the context of primary and secondary prevention of CV events [24]. In Belgium in 2021, INAMI reports that drugs prescribed as part of the cardiovascular system treatment, rank second in terms of the gross amount (€) attributed to that domain and are predominantly first when counting the defined daily dose (DDD) per 1000 inhabitants per day (Figure 1.3A, panels (a) and (b)). In the cardiovascular system category, the top three drugs with the highest number of delivered DDD in 2021 are statins (584,060,902 doses), hypotensive medication (247,244,000 doses), and beta-blockers (187,829,000 doses). Among statins, INAMI also reports the large prevalence of ATV among the prescriptions since 2015, as represented on Figure 1.3B. While pravastatin and fluvastatin prescription remained the lowest over the years, the trend fluctuated between ATV, rosuvastatin and simvastatin. Simvastatin was indeed associated with the highest DDD until 2015, before being overtaken by ATV that year and by rosuvastatin since 2019 [35]. In the United States, ATV also holds the highest prescription rate among statins, with simvastatin following as

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the second most prescribed [36]. The prevalence of ATV prescriptions in Europe and overseas justifies the study of this drug in our project.

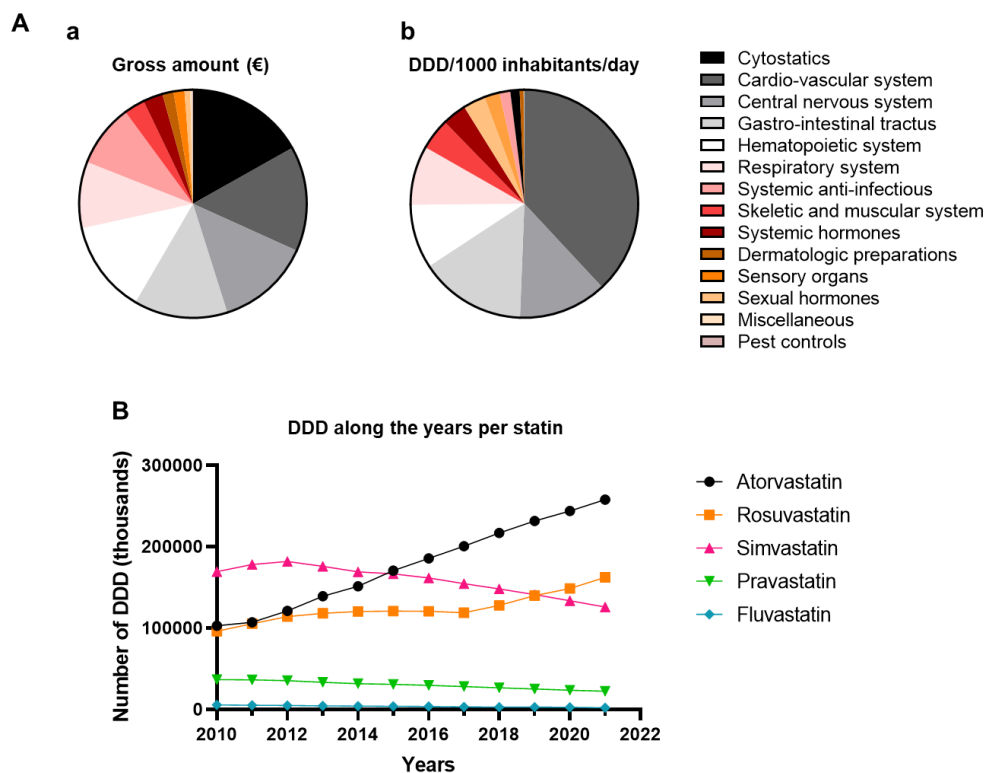


Figure 1.3. INAMI prescription statistics by all approved prescribers. (A) Pie charts of drug prescriptions classified by anatomical levels represented (a) by total gross amount (€) attributed by categories and (b) in DDD per 1000 inhabitants per day. (B) Number of DDD (thousands) prescribed along the years per statin.

2. Atorvastatin

Some parts of this section have been published as a review in Clinical Biochemistry in January 2024.

A. Mechanism of action

a) Therapeutic effects

Atorvastatin, including all statins, competitively inhibit the HMG-CoA reductase with an affinity of 1000 to 10,000-fold more important than that of the endogenous substrates like essential fatty acids (e.g. γ -linolenic acid, arachidonic acid, prostaglandins E1) [37]. The first step of the cholesterol synthesis, which leads to the formation of HMG-CoA, occurs in the cytosol of the hepatocytes whereas the following steps take place in the endoplasmic reticulum of the hepatic cells [38]. HMG-CoA reductase inhibition leads to the reduction of the cholesterol synthesis, directly contributing to the LDL-C lowering effect. The direct intracellular reduction in cholesterol content in the hepatocyte cells, will activate SREBP-2 that, when upregulated, increase the LDL-receptors at the hepatocyte cell membranes increasing the clearance of LDL-C from the blood [39, 40].

As illustrated on Figure 1.4, the inhibition of the cholesterol synthesis is, however, the last step of the mevalonate pathway indicating that its inhibition also includes a depletion of the upstream cholesterol precursors and other mevalonate downstream metabolites such as dolichol, prenylated proteins, isoprenoids and ubiquinone or Coenzyme Q10 [41, 42].

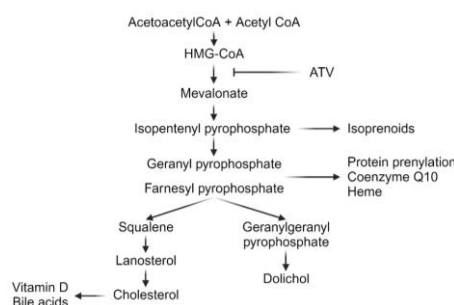


Figure 1.4. Mevalonate pathway and ATV inhibition. ATV inhibits the HMG-CoA reductase catalyzing the formation of mevalonate, to *in fine* reduce the production of cholesterol. Created with BioRender.com

b) Pleiotropic effects

The effects of statins are therefore LDL-C dependent due to their direct lipid-lowering impact as well as LDL-C independent resulting from their pleiotropic other effects [43]. Those are numerous and may be the consequences of the reduced intermediates of the mevalonate pathway such as the isoprenoids. They are implicated in the isoprenylation of Rho, involved in the formation of reactive oxygen species leading to cardiac hypertrophy. Logically, statins were associated with a protective effect against cardiac myocytes hypertrophy by inhibition of the formation of isoprenoids [44]. Statins pleiotropic effects may also arise from other pathways like the inhibition of Nox2 after ATV administration, acting as an antioxidant which was simultaneously associated with an antiplatelet response via the inhibition of platelet isoprostane and Thromboxane B₂. This mechanism that adds another layer to the prevention of coronary thrombosis was observed in 30 patients suffering from polygenic hypercholesterolemia and confirmed *in vitro* [45]. On the contrary, another study failed to demonstrate any benefit from the antioxidant effect of statins on atrial fibrillation after cardioversion [46]. Another illustration of statins' pleiotropy is their implication in reducing the inflammation that accompanies atherosclerosis. It has indeed been demonstrated in various studies that ATV not only reduces the cholesterol deposition in the artery walls, but also directly inhibits the NLRP3 inflammasome and toll like receptors, promoting the anti-inflammatory response. This effect was mostly observed for the lipophilic statins, as, for instance, hydrophilic fluvastatin was shown to be associated with an amplification of the inflammation [47].

The pleiotropic effects of statins are thus double-edged. On the one hand, they show numerous beneficial effects and, on the other, they promote deleterious myotoxic effects mostly resulting from the depletion of the mevalonate pathway metabolites that are unable to achieve their physiological roles in the cells, as depicted in the following paragraph [48, 49]. This observation is underscored by the prolonged duration required for the establishment of beneficial effects related to statins. Conversely, if a patient abruptly ceases the intake of the medication, the side effects manifest rapidly increasing their acute ischemic risk.

B. Atorvastatin-related side-effects

Even though statins are relatively well-tolerated, 7 to 15% of patients suffer from statin-induced skeletal muscle abnormalities or complains, ranging from benign myalgia to rare but potentially life-threatening rhabdomyolysis [50, 51]. Commonly reported muscular side-effects, subsumed under the umbrella of statin-related myotoxicity (SRM) or statin-associated myopathy (SAM), appear under various forms. In practice, the most frequent muscular event observed with statin use consists of myalgia or moderate myopathy (Table 1.1). If signs of intolerance are experienced, it is advised to switch therapy. Nonetheless, switching therapy is not always enough to improve symptoms and, in the worst scenario, this can lead to necrotizing autoimmune myotoxicities [52].

There is, at present, no real consensus on the definition of SAM or SRM. According to the American College of Cardiology (ACC), the term myopathy stands for any muscular disease while myalgias are commonly defined by muscle aches, cramps, or complaints with normal creatine kinase (CK) levels. Myositis refers to muscular symptoms with an elevation in CK but not higher than 10 times the upper limit of normal (ULN) range [53]. Finally, rhabdomyolysis is diagnosed by the presence of the precited symptoms with a CK elevation above 10 times the ULN and creatinine elevation with brown urine and urinary myoglobin [54, 55]. The Food and Drug Administration (FDA) definitions do not include myositis as a separate category and defines rhabdomyolysis as CK of > 50 times the ULN with renal function alterations [56]. The European Phenotype Standardization Project (EPSP) has also defined SRM phenotypes with slight differences compared to ACC or FDA definitions (Table 1.1). For the sake of clarity and because there is no real consensus on the definition of the toxicity endpoints investigated in clinical studies, the term SRM will be used throughout this manuscript to reflect any muscular event/outcome whatever the original definition used in the original study considered. Even if this oversimplification does not reflect the exact EPSP definition of SRM, most clinical studies evaluate SRM based on self-reported muscle symptoms or symptomatology only and CK levels are rarely measured. Thereby, the work focuses mainly on common muscular side-effect and considers mainly myalgia (SRM1 and 2) and possibly moderate and few severe myopathy (SRM 3 and 4).

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Statin-related myotoxicity phenotype classification.		
SRM classification	Phenotype	Clinical assesement
SRM 0	CK elevation <4 x ULN	No muscle symptoms
SRM 1	Myalgia, tolerable	Muscle symptoms No CK elevation
SRM 2	Myalgia, intolerable	Muscle symptoms, complete resolution on dechallenge CK <4 x ULN
SRM 3	Myopathy	Muscle symptoms, complete resolution on dechallenge CK elevation >4 x ULN <10 x ULN
SRM 4	Severe myopathy	Muscle symptoms, complete resolution on dechallenge CK elevation >10 x ULN <50 x ULN
SRM 5	Rhabdomyolysis	Muscle symptoms + CK elevation >10 x ULN with evidence of renal impairment OR CK >50 x ULN
SRM 6	Autoimmune-mediated necrotizing myositis	HMGCR antibodies, HMG-CoA reductase expression in muscle biopsy, incomplete resolution on dechallenge
Adapted with permission from Alfirevic <i>et al.</i> [57]		

Table 1.1. Distinction of the six levels of SRM characterized by the patients' phenotype and the clinical assessment.

Apart from impacting on patients' adherence (developed in the following section), SRM also impact on quality of life of the patients, which is particularly inconvenient considering chronic medications. Unfortunately, the risk of side-effects for a given *de novo* patient remains at present unpredictable. There are multiple risk factors for SRM including patient-specific (age, genetics, weight...) or drug-related (metabolism, transport, dose, drug-drug interaction...) factors but the associations are not always well-defined. It is, however, established that SRM are dose- and concentration-dependent and reversible upon discontinuation, suggesting that differences in patients' tolerance would have a pharmacokinetic (PK) basis [58, 59]. Deciphering the reasons for PK variabilities and more particularly, local PK within the muscle tissue, might help in modulating the risk of muscle

side effects in each patient and has the potential to optimize patient adherence and quality of life on the long term.

A meta-analysis assessing the efficacy of statins, however, did not find an association between the treatment and muscle complaints. However, the strict inclusion and exclusion criteria of a randomized clinical trial primarily centered around efficacy, can clarify this absence of observed side effects [60]. Also, this negative result could be attributed to the analysis that was carried out on all statins combined, at different doses.

C. Adherence to the treatment

As for any chronic treatment, the effectiveness of statin therapy largely depends on patients' adherence. Adherence is defined by the WHO as *"The extent to which a person's behavior – taking medication, following a diet, and/or executing lifestyle changes, corresponds with agreed recommendations from a health care provider"* [61]. Furthermore, the European Society for Patient Adherence (ESPACOMP) divides adherence into three distinct phases: (i) initiation, that refers to the first dose, (ii) implementation, defined as *"the extent to which a patient's actual dosing corresponds to the prescribed dosing regimen from initiation until the last dose is taken"*, and (iii) persistence, that expresses the time between initiation and the last dose. Issues can occur in any of these phases. Regarding statin therapy, early discontinuation (or non-persistence) but also poor adherence (e.g. dose skipping/drug holidays) represents a major problem. Discontinuation refers to patients stopping the medication on their own initiative, taking no dose thereafter whereas non-adherence more commonly refers to the reciprocal of adherence [62]. Adherence to statin therapy is suboptimal in both primary and secondary prevention of CVD. The exact rate of non-adherence is difficult to determine. However, a meta-analysis highlighted a global adherence rate to statins of 49% among observational studies and of 90.3% in randomized trials in patients after 1 year of treatment [63]. Several sources of non-adherence have been identified (described in Table 1.2), among which side-effects is one of the leading causes [25]. As SRM is the most common statin side-effect and represents an important cause of statin intolerance or patient discomfort, it can lead to poor adherence and/or spontaneous drug discontinuation, which majours the risk of treatment failure [64, 65]. Contrarily, patients

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suffering from co-morbidities, such as high blood pressure or diabetes, were associated with a significant increase of statin adherence [65, 66].

Causes of non-adherence to statin treatment	
Self-perceived factors	SRM development [25, 65]
	Lack of perceived benefits in primary prevention [25, 66]
Educational factors	Media coverage of the side-effects [25]
	Physicians' explanation of statin treatment [65]
	Chronic treatment [61]
Sociodemographic factors	African ethnicity [65]
	Household income < 50.000\$/year [65]

Table 1.2. Main causes of non-adherence to statin treatment.

D. Putative mechanisms of atorvastatin-associated myotoxicity

There are numerous hypotheses to explain the muscular toxicity observed with statins, but none is sufficient to elucidate the underlying physiopathology, which remains speculative at this stage. However, dressing up the general pathophysiologic basis of SRM appears important to understand the role of PK modulations in the development of muscle side effects. The most convincing hypothesis on the etiology of SRM suggests that the inhibitory effect of ATV on the mevalonate pathway within the muscle and the subsequent depletion in intermediate derived metabolites are responsible for the observed muscle toxicity. *In vitro* studies show that blocking cholesterol synthesis by inhibiting squalene production (which appears distally in the pathway) does not cause muscle toxicity [67]. In rat skeletal muscle cells, squalene supplementation is not able to reverse muscle toxicity caused by statins whereas statin-induced myotoxicity is reversed when precursors of upper isoprenoid intermediaries are given [67]. This assumption is further supported by the fact that muscle-related side effects of statins are dose-, potency- and concentration-dependent. The vulnerability of skeletal muscle compared to other tissues might be explained by the heightened responsiveness of muscle cells to the statin-induced inhibition of cholesterol synthesis, as reflected by a lower IC₅₀ [58, 68]. Indeed, *in vitro*, it has been demonstrated that the IC₅₀ for ATV HMG-CoA reductase inhibition in human skeletal muscle-cells is 44.5

times lower than in human primary hepatocyte cells (IC_{50} values of 4.7nM and 209nM, respectively) [68]. However, as the IC_{50} is highly dependent on the experimental conditions, and since the HMG-CoA reductase isoforms are similar in all tissues, this IC_{50} difference is likely due to a differential environment rather than differential affinities for the enzyme. Among intermediate metabolites, isoprenoid compounds like geranylgeranyl pyrophosphate and farnesyl pyrophosphate are responsible for protein prenylation [69], among which small GTPases like Rho, Ras and Rab. Rho, Ras and Rab are activated upon isoprenylation and further regulate vital cell functions such as cell integrity, division, differentiation, fusion and trafficking, therefore playing key roles in cell survival [70, 71]. On the contrary, a reduced isoprenylation of the intermediate geranylgeranyl induces the synthesis of atrogen-1, a muscle-specific F-box protein playing a key role in muscle atrophy [72, 73]. The increased secretion of this protein is thought to influence the development of muscle-related side effects. It was indeed demonstrated that not only the atrogen-1 expression is significantly increased in skeletal muscle biopsies of patients suffering from SRM but also that knocking down the expression of the protein in zebrafish embryos, prevent them from developing lovastatin-induced SRM [74]. Additional evidence supporting the idea that isoprenylation may be responsible for the observed muscle toxicity of statins comes from the observation that rare inborn mevalonate kinase deficiency is associated with, among other, congenital defects myopathy whereas mutations in distal enzymes of the cholesterol synthesis (that do not affect prenylation) are not [75]. The inhibition of the HMG-CoA reductase has also the consequence to reduce the generation of intermediary products such as dolichol and ubiquinone, also known as coenzyme Q10. The former is implicated in oligosaccharides N-glycosylation [76] while the latter is critical for the electron transport chain of the mitochondrial respiration [77]. Statins may thus interfere with mitochondrial function, causing an impaired muscle performance, resulting in muscle damages observed in patient suffering from SRM [69, 78]. Nevertheless, supplementing patients suffering from SRM with coenzyme Q10, did not modify the resolution of the muscle complaints compared to patients who did not receive supplementation [79].

In summary, the pathophysiology of SRM is inevitably complex because of the long list of affected intermediates in the mevalonate pathway and the subsequent changes in cellular

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functions. Most of the elaborated scenarios link SRM events to the inhibition of the mevalonate pathway within the muscle cell, highlighting the importance of local drug exposure for explaining SRM individual susceptibility.

E. Atorvastatin pharmacokinetics and ADME overview

The daily recommended ATV dose is 10-80mg taken orally as a calcium salt of the active acid form in the morning or evening, outside the meals. ATV is characterized as a moderate- to high-intensity statin depending on the dose taken, it will induce 30-49%, or more than 50% reduction in LDL-C [30]. It is rapidly and well absorbed but has a weak and variable oral bioavailability of approximately 12% due to substantial first-pass metabolism [80]. Once ingested, ATV crosses the gut barrier mainly by passive diffusion with only a small fraction (30%) of this lipophilic drug permeating via uptake transporters [80]. A fraction of the drug that has crossed the apical membrane is re-expelled into the gut lumen via efflux transporters while remaining fraction is transported to the hepatocytes via the portal vein to be metabolized [81].

ATV is known to be a substrate of uptake transporters from the solute carrier organic anion (*SLCO*) family coding for organic anion-transporting polypeptide (OATP); *SLCO1A2* (OATP1A2), *SLCO1B1* (OATP1B1), *SLCO1B3* (OATP1B3) and *SLCO2B1* (OATP2B1) [82, 83]. ATV PK is also modulated by ATP-binding cassette efflux transporter (*ABC*) superfamily members including *ABCB1* (P-glycoprotein or P-gp), *ABCC1* (MRP1), *ABCC2* (MRP2), *ABCC4* (MRP4) and *ABCG2* (BCRP) [81].

a) Solute carrier transporter family

Solute carrier proteins (SLC) is the largest transporter super family counting more than 400 proteins divided into 52 families, that mediate the transport of various molecules into the cells [84]. This transporter super family includes all membrane proteins that transport nutrients, metabolites, ions or xenobiotics across membranes in a facilitative or secondary active way to maintain the body homeostasis [85]. The facilitated transport consists of substrate transportation across membranes via protein carriers from a high to a low concentration gradient. Whereas secondary active transport is the transport of a compound

in the direction of its increasing electrochemical potential coupled to the transport of an ion in the direction of its decreasing electrochemical potential [86, 87]. This family gathers various transporters categories issued from SLC subfamilies. Five of the most commonly known subfamilies are described in Table 1.3 [88].

Subfamilies	Transporter families	Major subfamily members	General characteristics	Principal localizations
<i>SLC21</i> gene subfamily	Organic anion transporters: OATPs	OATP1A2, OATP1B1 , OATP1B3 , OATP1C1, OATP2A1, OATP2B1 , OATP3A1, OATP4A1, OATP4C1, OATP5A1, OATP6A1	Transport large and fairly hydrophobic organic anions	Liver, brain, testis [89]
<i>SLC22A</i> gene subfamily	Organic anion transporters: OATs and urate transporters: URAT1	Branches; A: OAT1, OAT3; B: OAT6; C subgroup D: OAT2, OAT10; C subgroup E: OAT5, OAT7, OAT8, OAT9; C subgroup F: OAT4 and URAT1	Transport the smaller and more hydrophilic organic anions	kidney, liver, choroid plexus, olfactory mucosa, brain, retina, and placenta [90]
	Organic cation transporters: OCTs	OCT1, OCT2, OCT3, OCT6	Transport organic cations	Ubiquitous but more importantly in postsynaptic neuronal and glial cells [91]
	Organic cation/carnitine transporters: OCTNs	OCTN1, OCTN2, OCTN3	Electroneutral organic cation transporters	Brain, bone marrow, skeletal muscle tissue [92, 93]
<i>SLC15A</i> gene subfamily	Peptide transporters: PEPTs	PEPT1, PEPT2, PHT1, PHT2	Uptake of di-/tripeptides and peptide-like drugs	Brush-border membranes of intestinal and renal epithelial cells [94]

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<i>SLC16</i> gene subfamily	Monocarboxylate transporters: MCTs	MCT1 to MCT14	Proton-dependent transporters	Ubiquitous [95]
<i>SLC47A</i> gene subfamily	Multidrug and toxin extrusion: MATEs	MATE1, MATE2, MATE2-B, MATE2-K	Efflux transporters of organic cations	Kidney, adrenal gland, choroid plexus [92, 93]

Table 1.3. Most commonly known SLC subfamilies. The members, their principal characteristics and localizations are described [88, 95].

ATV absorption and cellular uptake are favored by OATPs expressed along the gastrointestinal tract and the liver as well as the ubiquitously expressed monocarboxylate transporters belonging to the proton-linked plasma membrane transporters family. MCT1 (*SLC16A1*) and MCT4 (*SLC16A3*) that are members of this subfamily, are mostly expressed in the oxidative fibers and in the glycolytic fibers of the skeletal muscle tissue, respectively [96, 97].

In this project, the focus was set on OATPs, and more specifically on OATP1B1, OATP1B3 and OATP2B1, as they are known to mediate xenobiotics transportation as well as numerous hydrophobic organic anions. They are also regularly highlighted in pharmacogenetic and PK studies related to the use of statins. The location and the most common substrates and inhibitors for each of the influx transporters are depicted in the Table 1.4. OATP1A2 will not be further investigated as it is mainly localized in the BBB and has barely been studied for its implication in ATV transport [98].

Proteins	Localization	Substrates	Inhibitors
SLCO1B1	Sinusoidal hepatocyte membranes [99, 100]	Bile salts, bilirubin glucuronides, thyroid hormones and steroid hormone metabolites. Statins , HIV protease inhibitors, anticancer agents (irinotecan, methotrexate,	5-carboxyfluorescein diacetate (CFDA), coumetrol, nifedipine, cyclosporine, chloroquine [103, 104]

		docetaxel), rifampicin, MPAG [100-102]	
SLCO1B3	Sinusoidal hepatocyte membranes [105]	Rifampicin, olmesartan, methotrexate, docetaxel, digoxin, pioglitazone, MPAG, statins , warfarin [101, 102, 106, 107]	Clarithromycin, cyclosporine, azithromycin, erythromycin, rifampicin, nateglinide, roxithromycin, tolbutamide and CFDA [88, 103]
SLCO2B1	Liver, enterocytes lumen, BBB, kidneys, smooth and skeletal muscle tissue, lungs [108-110]	Estrone-3-sulfate, dehydroepiandrosterone sulfate, dibromofluorescein, montelukast, bromosulfophthalein, statins , atenolol, azilsartan, diclofenac, [111, 112]	Erlotinib, L-thyroxin, cyclosporin A, gemfibrozil, angiotensin II receptor blockers, KO143, atorvastatin [103, 113-115]

Table 1.4. SLCO transporters localization, substrates and inhibitors.

The OATP sub-category contains 11 sodium-independent influx transporters classified on their homology in sequences, see Table 1.3. All OATP members are constituted of 12 membrane-spanning domains (MSD) with the third and the fifth extracellular loops being glycosylated and both N- and C- terminal cytosolic ends. They also contain a signature sequence region overlapping on the third extracellular loop and the sixth MSD [88]. This highly conserved sequence has been demonstrated to be implicated in the protein expression at the membrane as the protein expression at the cell surface was significantly reduced by point mutations in the signature sequence of OATP1B1 [116].

OATP transporters' expression is regulated by various coordinated mechanisms. *OATP1B1*, *OATP1B3* and *OATP2B1* regulation can be done by DNA methylation or histone modification. The nuclear receptors farnesoid X factor (FXR) and hepatocyte nuclear factor (HNF) also regulate the expression of *OATP1B1* and *OATP1B3* in the liver. Whereas pregnane X factor (PXF) regulates the expression of the three proteins. Constitutive androstane receptor (CAR) secretion would be associated with a decreased expression of *OATP1B3* and

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OATP2B1. Whereas aryl hydrocarbon receptor (AhR), retinoid X receptor (RXR) and retinoic acid receptor (RAR) would be implicated in *OATP1B1* and *OATP2B1* regulation. All the factors coordinate and upregulate each other, in the case of *OATP1B3*, FXR secretion will upregulate CDCA and DCA that will, as a consequence, upregulate HNF (1 α and 3 β) [89, 106, 117].

- ***SLCO1B1***

OATP1B1, or *SLCO1B1*, is an influx transporter composed of 691 amino acids encoded by 15 exons on chromosome 12p12.1. The *SLCO1* gene locus contains *SLCO1B1* and also three other genes from this family; *SLCO1B3*, *SLCO1B7* and *SLCO1A2*. In the locus, *SLCO1B1* is localized between *SLCO1B7* and *SLCO1A2* and shows 80% identity with *SLCO1B3* [104, 118]. The expression of *SLCO1B1* and *SLCO1B3* was indeed correlated in the study presented in Chapter IV. *OATP1B1* transporter has a molecular weight of 84kDa and is very specifically and highly expressed on the basolateral hepatocytes membranes, participating to the uptake of drugs to be metabolized or excreted in this tissue [99]. This protein transports endogenous substrates such as bile salts, bilirubin glucuronides, thyroid hormones and steroid hormone metabolites but also xenobiotics such as statins, HIV protease inhibitors and anticancer agents (irinotecan, methotrexate) [100]. As indicated in Table 1.4, chloroquine has been identified as an inhibitor of *OATP1B1*. The drug inhibits *OATP1B1* degradation by retaining the protein in the lysosomes, reducing the hepatocyte *OATP1B1*-mediated transport of statins and as a result increasing the risk of SRM development [104, 119].

Due to the high expression of *SLCO1B1* in the liver, the gene has largely been studied for the implication of single nucleotide polymorphisms (SNP) in its sequence on the drug PK. The Table 1.5 gathers the most relevant coding SNPs affecting the gene; they are either highly expressed in the population, relevant in a haplotype (Figure 1.5) or studied for modulating the transport of drugs. The relevant non-coding SNP can be found in the Chapter VII – Annexes (Table 7.1).

SNPs	Frequency	<i>In vitro and/or in vivo effect</i>
rs2306283 (1) (c.388A>G, p.Asn130Asp)	AFR: 0.774 EAS: 0.752 SAS: 0.476 EUR: 0.403	↑ or ↓ <i>in vitro</i> function depending on the substrate [104, 120] No association with ATV or simvastatin lipid-lowering response [121] Significant ↑ of LDL-C reduction with ATV in GG vs AA/AG [122] ↓ ezetimibe AUC, no effect on its lipid-lowering effect [123] ↓ tacrolimus blood concentrations in heterozygous, no change in homozygous [124] Randomized control trial on 1279 patients associated variant with ↑ methotrexate clearance [125] ↓ ATV plasma concentrations with variant [126]
rs11045818 (2) (c.411G>A, p.Ser137=)	AFR: 0.036 EAS: 0.000 SAS: 0.043 EUR: 0.161	No association with ATV or simvastatin lipid-lowering response [121]
rs11045819 (3) (c.463C>A, p.Pro155Thr)	AFR: 0.077 EAS: 0.000 SAS: 0.043 EUR: 0.160	Unchanged <i>in vitro</i> functions [120]. No association of the variant with ATV response [122]
rs4149056 (4) (c.521T>C, p.Val174Ala)	AFR: 0.030 EAS: 0.125 SAS: 0.050 EUR: 0.159	Associated with decreased <i>in vitro</i> OATP1B1 activity [120] and a lower drug clearance [127] With statins ↑ AUC, C_{max} and SRM development (see section pharmacogenetics) [51, 128-132] Randomized control trial on 1279 patients associated the variant with ↑ methotrexate clearance [125] ↓ docetaxel transport [102] ↑ ATV plasma concentrations with variant [126]
rs4149057 (5) (c.571T>C, p.191Leu=)	AFR: 0.204 EAS: 0.255 SAS: 0.488 EUR: 0.618	43% irinotecan clearance ↓ in WT vs homo- and heterozygous in patients with body surface area ≤1.88 m² [133] Associated with ↑ breast cancer risks in a prospective study achieved on 133,479 females [134]
rs2291075 (6) (c.597C>T, p.199Phe=)	AFR: 0.552 EAS: 0.497 SAS: 0.211 EUR: 0.396	Tacrolimus C/D ratio significantly ↓ in homo- and heterozygous than in WT carriers in week 1 and 2 post-op [135]. Homozygous and, in a lesser extent, heterozygous carriers are associated with ↑ survival in 232 <i>de novo</i> pediatric patients with acute myeloid leukemia [136] Variant associated with ↓ percentage of monomethylarsonic acid and ↑ of dimethylarsinous acid percentage [137]

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rs59502379 (7) (c.1463G>C, p.Gly488Ala)	AFR: 0.036 EAS: 0.000 SAS: 0.000 EUR: 0.000	Induces a ↓ function <i>in vitro</i> [120], illustrated by ↓ docetaxel transport alone and an absence of transport in haplotype <i>SLCO1B1</i> *31 [102] In haplotype <i>SLCO1B1</i> *46, associated with the Rotor syndrome (benign hereditary conjugated hyperbilirubinemia) [104, 138]
rs71581941 (8) (c.1738C>T, p.Arg580Ter)	AFR: 0.001 EAS: 0.004 SAS: 0.001 EUR: 0.001	The variation induces a stop codon, found in the haplotypes *45 and *46 [104, 139-142]
rs34671512 (9) (c.1929A>C, p.Leu643Phe)	AFR: 0.066 EAS: 0.001 SAS: 0.045 EUR: 0.052	Associated with unchanged <i>in vitro</i> transport of sulfobromophthalein, estradiol-17β-D-glucuronide, pravastatin, and taurocholic acid in HEK293 transfected cells [120, 143]

Table 1.5. Relevant coding SNPs in *SLCO1B1*. Each SNP is identified by rs number and located in both the mRNA and in the protein. The frequency data are issued from gnomAD v2.1.1 and Ensembl [139-142]. AFR: African, EAS: East Asian, SAS: South Asian, EUR: European and AMR: American.

Some of the variants highlighted in Table 1.5 are relevant when studied alone, such as rs4149056 (c.521T>C, p.Val174Ala) that is also referenced as *SLCO1B1**5 (Figure 1.5). However, some are unique and only relevant when studied as part of a haplotype, such is the case of the SNP rs72559747 (c.1007C>G, p.Pro336Arg) found in the haplotype *SLCO1B1**47 constituted of two other SNPs (rs4149056 and rs2306283) [104, 144]. The SNP rs4149056 (c.521T>C) and rs2306283 (c.388A>G) are in linkage disequilibrium and are common to the haplotypes *5, *15 and *47. The rs4149056 has been highlighted as the key SNP that reduces the activity in the three haplotypes (Figure 1.5) [145, 146]. While the variant rs2306283 was found to be associated with a reduced response to ATV, the SNP rs11045819 did not modify ATV response. However, when part of the haplotype *14, they were associated with a decreased ATV AUC in a study led on 82 volunteers, compared to the referenced haplotype [100].

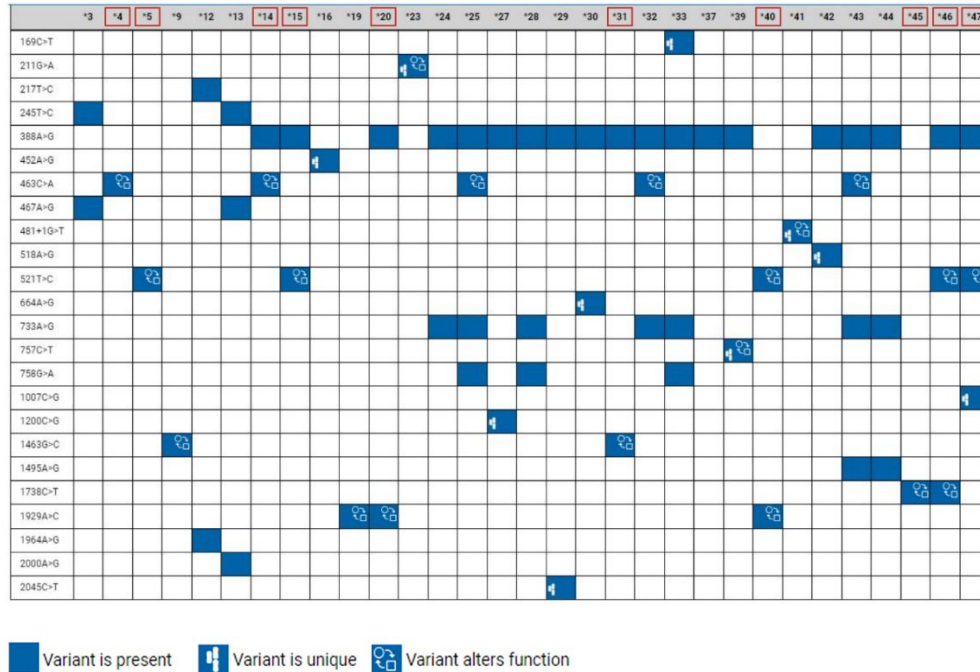


Figure 1.5. Most relevant haplotypes in *SLCO1B1* obtained from the Pharmacogene Variation Consortium (PharmVar) [104, 144]. The haplotypes containing the SNPs highlighted in Table 1.5 are framed in red.

Three of the relevant polymorphisms presented in Table 1.5 will be detailed in the section “Sources of variability influencing atorvastatin pharmacokinetics and muscular toxicity” of this manuscript as they have been described to significantly impacts ATV PK and the development of statin-related side effects. Those are the SNPs rs2306283, rs11045818 and rs4149056 in *SLCO1B1* sequence, identified by the numbers (1), (2) and (4) on the Figure 1.6A, respectively. Both SNPs rs2306283 (1) and rs11045818 (2) are localized in the same second extracellular loop. rs2306283 (1) is non-synonymous and induces the replacement of an uncharged by a negatively charged amino acid. The loss of function induced by the non-synonymous SNP rs4149056 (3) could supposedly come from alanine being one carbon junction shorter than valine and oriented inside the substrate channel. This could alter the interaction with the protein’s substrates and reduce its activity.

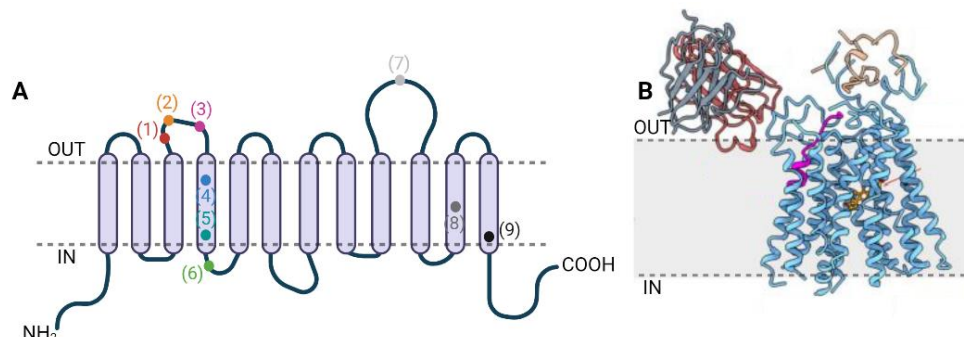


Figure 1.6. OATP1B1 topology model (A) and tertiary structure (B). (A) Topology of the protein including the relevant coding SNPs indicated by numbers referring to Table 1.5. (B) The tertiary structure is adapted from Ciută et al [147].

- **SLCO1B3**

OATP1B3 is composed of 702 amino acids spread in 16 exons on chromosome 12p12.2. The glycosylated protein has a molecular weight of ~100kDa and transport various anionic compounds comparable to OATP1B1. The protein is essentially localized on the sinusoidal membrane of hepatocytes [105]. The Table 1.6 gathers the most relevant coding SNPs affecting the gene; they are either highly expressed in the population, relevant in a haplotype or studied for modulating the transport of drugs. The relevant non-coding SNP can be found in the Chapter VII – Annexes (Table 7.2).

SNPs	Frequency	<i>In vitro</i> and/or <i>in vivo</i> effect	
rs4149117 (1) (c.334T>G, p.Ser112Ala)	AFR: 0.426 EAS: 0.725 SAS: 0.905 EUR: 0.851	Unchanged <i>in vitro</i> transport function [120, 148]. Not significantly associated with time to progression in androgen deprivation therapy [149] See rs7311358 for effects in haplotype	No impact of the haplotype on ATV transport [148]
rs57585902 (2) (c.439A>G, p.Thr147Ala)	AFR: 0.031 EAS: 0.000 SAS: 0.000 EUR: 0.000	No impact on docetaxel or cholecystokinin-8 transport <i>in vitro</i> [102, 148]	
rs60140950 (4) (c.767G>C, p.Gly256Ala)	AFR: 0.026 EAS: 0.001 SAS: 0.070 EUR: 0.157	Associated with 22% ↑ telmisartan AUC _{0-∞} per copy of the minor allele in 188 volunteers [150]	

rs2043949386 (5) (c.1559A>C, p.His520Pro)	na	Significant cholecystokinin-8 and rosuvastatin ↓ transport <i>in vitro</i> [148]	
rs7311358 (3) (c.699G>A, p.Met233Ile)	AFR: 0.436 EAS: 0.720 SAS: 0.869 EUR: 0.853	Significant cholecystokinin-8 ↓ transport <i>in vitro</i> and in 334G-699A haplotype [148] Haplotype 334G-699A induces a ↓ MPAG uptake <i>in vitro</i> with a ↓ V _{max} [101] an identical ↓ uptake was observed with testosterone [151] ↓ docetaxel transport both alone and in haplotype 334G-699A but less significantly [102] Haplotype with rs4149117 and rs7311358 variant carriers associated with ↓ warfarin mean daily dose [107]	
rs72559743 (6) (c.1564G>T, p.Gly522Cys)	AFR: 0.000 EAS: 0.000 SAS: 0.000 EUR: 0.000	Reduced transport activity <i>in vitro</i> [146, 152]	
rs12299012 (7) (c.1679T>C, p.Val560Ala)	AFR: 0.026 EAS: 0.000 SAS: 0.000 EUR: 0.000	↓ docetaxel, cholecystokinin-8 and rosuvastatin transport <i>in vitro</i> [102, 146, 148]	

Table 1.6. Relevant coding SNPs in *SLCO1B3*. Each SNP is identified by rs number and located in both the mRNA and in the protein. The frequency data are issued from gnomAD v2.1.1 and Ensembl [139-142]. AFR: African, EAS: East Asian, SAS: South Asian, EUR: European and AMR: American.

The Figure 1.7a includes all the relevant coding SNPs in *SLCO1B3* described in Table 1.6. The four SNPs rs4149117, rs57585902, rs60140950 and rs2043949386 forming a haplotype and localized on the Figure 1.7A by the numbers (1), (2), (4) and (5) will be further detailed in the section “Sources of variability influencing atorvastatin pharmacokinetics and muscular toxicity”. The SNP rs60140950 (4) induce the replacement of the first amino acid of the sixth MSD which interfere with the signature sequence region DSRWV//GAWWLGF_L that overlaps on the third extracellular loop and the sixth MSD. Its minor allele frequency is rather low but has been associated with telmisartan transport alteration, increasing its AUC_{0-∞}, which suggests a reduced influx transport activity mediated by OATP1B3 [150].

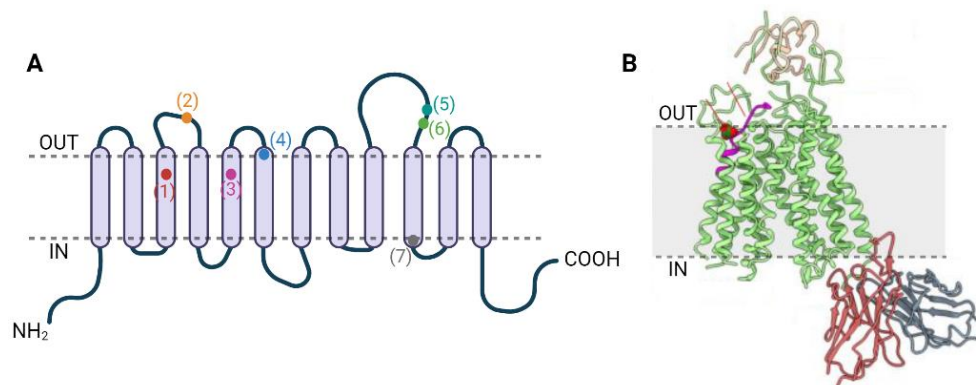


Figure 1.7. OATP1B3 topology model (A) and tertiary structure (B). (A) Topology of the protein including the relevant coding SNPs indicated by numbers referring to Table 1.6. (B) The tertiary structure is adapted from Ciută *et al* [147].

- **SLCO2B1**

Organic anion-transporting polypeptides subfamily member 2B1, or OATP2B1, is an influx transporter containing 709 amino acids. They are encoded by 14 exons in chromosome 11q13.4. This protein of 82kDa is expressed in a wide variety of tissues compared to OATP1B1 and 1B3. That includes the liver, the enterocytes lumen implicated in the absorption, the BBB, the kidneys and the smooth and skeletal muscle tissue [108-110]. This protein has been linked to the transport of many xenobiotics such as statins (ATV, rosuvastatin, fluvastatin, pravastatin, simvastatin), atenolol and diclofenac [112]. But also endogenous substrates like estrone-3-sulfate, dibromofluorescein, L-thyroxine and dehydroepiandrosterone sulfate as represented in Table 1.4 [111]. The Table 1.7 is a summary of the most relevant coding SNPs in *SLCO2B1* with their corresponding frequency and clinical significance. The relevant non-coding SNPs can be found in the Chapter VII – Annexes (Table 7.3).

SNPs	Frequency	<i>In vitro</i> and/or <i>in vivo</i> effect
rs142693902 (1) (c.332G>A, p.Arg111Gln)	AFR: 0.000 EAS: 0.000 SAS: 0.000 EUR: 0.001	Significant ↓ rosuvastatin, coproporphyrin III, dehydroepiandrosterone sulfate and estrone sulfate <i>in vitro</i> [153]

rs35199625 (2) (c.601G<A, p.Val201Met)	AFR: 0.003 EAS: 0.063 SAS: 0.039 EUR: 0.011	Significant ↓ dehydroepiandrosterone sulfate, rosuvastatin and coproporphyrin III <i>in vitro</i> [153]
rs12422149 (3) (c.935G>A, p.Arg312Gln)	AFR: 0.091 EAS: 0.326 SAS: 0.218 EUR: 0.104	Tendency of ↓ ATV concentrations in muscle [154] In 935A: slight [155] to significant [156] ↓ of SLCO2B1 ATV uptake ↓ montelukast plasma concentrations in 489 adults and confirmed on 26 teenagers [157, 158] but no significant effect on 16 young adults [159] ↑ dehydroepiandrosterone sulfate variant uptake <i>in vitro</i> [160]
rs1621378 (4) (c.1175C>T, p.Thr392Ile)	AFR: 0.000 EAS: 0.000 SAS: 0.000 EUR: 0.000	↓ estrone-3-sulfate <i>in vitro</i> transport [120, 161]
rs2306168 (5) (c.1457C>T, p.Ser486Phe)	AFR: 0.329 EAS: 0.250 SAS: 0.063 EUR: 0.024	↓ estrone-3-sulfate transport (V_{max}) more than half compared to WT <i>SLCO2B1</i> in HEK293 cells [161] Significant ↓ dehydroepiandrosterone sulfate, estrone sulfate, rosuvastatin and coproporphyrin I, <i>in vitro</i> [153] Not associated with warfarin dose in a quantitative analysis but well in a microarray screening [107]

Table 1.7. Relevant coding SNPs in *SLCO2B1*. Each SNP is identified by rs number and located in both the mRNA and in the protein. The frequency data are issued from gnomAD v2.1.1 and Ensembl [139-142]. AFR: African, EAS: East Asian, SAS: South Asian, EUR: European and AMR: American.

All five *SLCO2B1* coding SNPs are localized on the 2D structure of the protein of Figure 1.8a. The SNP rs12422149, numbered (3) on the Figure 1.8, is studied in the pharmacogenetics section below for its implication in ATV PK modulation. The polymorphism induces a non-synonymous variation in the third intracytosolic loop of the protein, localized after the signature amino sequence being DPRWV//GAWWLGL (3rd extracellular loop//6th MSD) [93, 116].

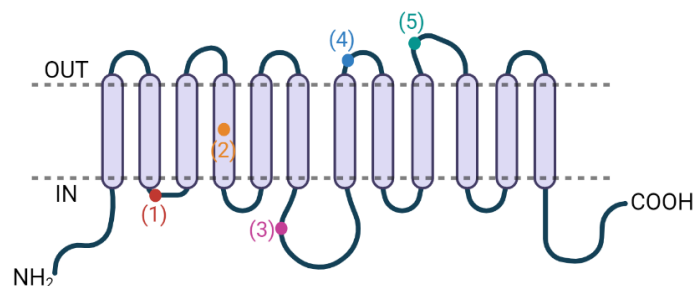


Figure 1.8. OATP2B1 topology model. Topology of the protein including the relevant coding SNPs indicated by numbers referring to Table 1.7.

Another characteristic of SLCO2B1 is that its mediated transport is pH dependent since a study with statins has shown that an acidic extracellular pH increases the import of the drug compared to a 7.4 pH [162]. This particularity has also been investigated by Shirasaka *et al.* The research group demonstrated in oocytes transfected with SLCO2B1, that the transporter has multiple binding sites for its substrate estrone-3-sulfate. They indeed showed a biphasic saturation with K_m values of 0.1 ± 0.05 and 29.9 ± 12.1 μM and V_{max} of 14.1 ± 6.4 and 995 ± 273 for the high and the low affinity binding sites, respectively. More importantly, they showed that the low affinity site is pH-dependent, being less efficient at higher pH whereas no change was observed with the high affinity binding site [163]. This characteristic could explain the variability observed between the different inhibitory studies that were performed [111].

b) ATP-binding cassette transporters

Human ATP-Binding Cassette (ABC) family members is composed of 49 efflux transporters divided into seven sub-families (from A to G), ubiquitously expressed in the human body [164]. The classification between the different sub-families is based on their gene structure, amino acid sequence, domain organization and phylogenetic analysis [165].

Some indeed only contain one transmembrane domain (TMD) followed by one nucleotide-binding domain (NBD), called half-transporters. Others are considered as full-transporters as they are composed of two TMD each one followed by a cytoplasmic NBD [166]. To be functional, ABC efflux transporters must be dimerized. Full transporters already form a dimerized polypeptide whereas half-transporters will have to fuse together. Consequently, their conformation is either oriented inward, allowing the substrates to bind to the transporter, or outward to expel the substrates out of the cells (Figure 1.9) [167]. ABC-proteins transport a wide variety of endogenous

substrates such as ions, lipids, hormones or neurotransmitters as well as exogenous substrates against their concentration gradient, requiring the use of ATP (depicted on Figure 1.10). The intracellular lipophilic substrates enter the efflux pump via the lower level of the transporter (black arrow) whereas the hydrophilic substrates will directly achieve the binding site via the transmembrane domains (brown arrow). Once bound, ATP molecules are recruited to provide the energy needed to expel the substrates out of the cells. The hydrolyzed ATP (ADP) is further released in the cytoplasm of the cells and the phosphate issued from the reaction is recycled for future transports. The underlying mechanism to which the NBDs hydrolyze ATP is based on the coupling of the two domains [166, 167].

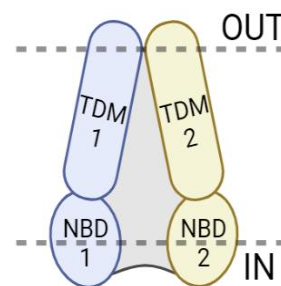


Figure 1.9. Inward dimerized ABC-transporter. Created by BioRender.com.

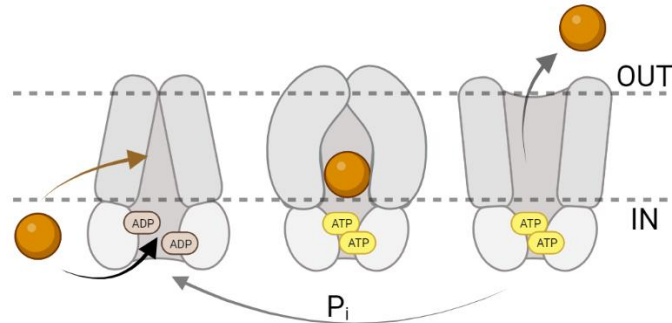


Figure 1.10. ABC-transport across membranes. Transport of lipophilic and hydrophilic substrates across membranes via ATP hydrolysis. “Out” corresponds to the extracellular compartment and “in” the cytoplasmic compartment. Created by BioRender.com.

While TMD sequences are very variable between the types of transporters and subject to genetic variations, NBD are composed of highly conserved motifs represented on the Figure 1.11. These conserved sequences are the Walker-A motif (also called P loop) that binds to the α - and β -phosphates of ATP, the Walker-B motif constituted of hydrophobic residues providing the catalytic glutamate, the Q-loop that provides contact to the TMD, the H-motif (or “switch histidine”) stabilizing the transition-state geometry, the A loop that provides an aromatic side chain and the D loop that is important for coupling the ATP hydrolysis to transport. Both loops (A and D) compose the catalytic core of the NBD which also include a α -helical domain less conserved that contains the ABC signature motif LSGGQ implicated in pinning and orientating ATP during hydrolysis [88, 167, 168]. The Walker A and Walker B motifs of one NBD subunit coupled with the Q-loop of the other NBD subunit to form the ATP-binding site where the two ATP molecules can bind and hydrolyze. In absence of ATP, the ABC protein is oriented inward with the two NBD separated from each other (Figure 1.9). As soon as ATP nucleotides bind one of the NBD, both domains are getting close to each other so that ATP gets stuck in between [88, 169].



Figure 1.11. NBD alignment in ABC transporters. In white is represented the full NBD sequence and in red the conserved motifs. Adapted from Liu X. [88] and created by BioRender.com.

In this work, based on previous observations (see below section “Sources of variability influencing atorvastatin pharmacokinetics and muscular toxicity”), the focus has been set on the sub-families B, C and G. More precisely, on the members ABCB1, ABCC1, ABCC2, ABCC4 and ABCG2 as they are relevant in the present manuscript regarding their substrate specificity or their localization as depicted in Table 1.8.

Proteins	Localization	Substrates	Inhibitors
ABCB1	Gastrointestinal tract, liver, BBB, kidneys, adrenal gland [170]	Anticancer drugs (bosutinib, dosatinib, erlotinib...), neurological agents (carbamazepine, citalopram, lamotrigine...), antibiotics, antihistaminic, statins , omeoprazole [88]	Verapamil, Cyclosporin A, vincristine, zosuquidar, tamoxifen, elacridar [88]
ABCC1	Ubiquitous [171]	Clozapine (neurological agent), doxorubicin, gemtuzumab, etoposide, irinotecan (anticancer), lamivudine, methotrexate (musculoskeletal agent, anticancer), vincristine (anticancer), taxane, statins [112, 156]	MK-571, S-decylglutathion, sulfinpyrazone, benzbromarone, probenecid but low cellular penetration [88]
ABCC2	Liver, kidney, gastrointestinal tract (duodenum and intestine) [172]	Anticancer drugs (methotrexate, doxorubicin...), HIV protease inhibitors (adefovir, indinavir...), toxins (bisphenol A...), other (statins , GSH, azithromycin...) [88]	MK-571, LTC ₄ , fluorescein methotrexate : competitive inhibitors [88]

ABCC4	Gastrointestinal tract, endocrine and male tissues, skeletal muscle tissue [173]	Acetaminophen, acyclovir, doxorubicin, fluoropyrimidine, ibuprofen, lamivudine, lamotirgine, methotrexate, tenofovir, nelfinavir, uricosuric, zidovudine, statins [112, 174]	MK-571, probenecid, nelfinavirinotecan [88, 175]
ABCG2	Gastrointestinal tract, liver, BBB, skeletal muscle tissue [176]	Anticancer drugs (anthracyclines, mitoxantrone, topoisomerase I inhibitors, topotecan, erlotinib, irinotecan...), anti-inflammatory (acetaminophen, diclophenac), HIV protease inhibitors, neurological agents (clozapine), other (statins , pantoprazole) [88, 112]	Elacridar, HIV protease inhibitors, tyrosine kinase inhibitors (e.g. erlotinib, nifitinib), novobiocin, cyclosporine, tacrolimus, angiotensin receptor antagonists, Ko143 [88, 177, 178]

Table 1.8. ABC transporters localization, substrates and inhibitors. They all are localized at the plasma membranes of the cells [179].

i. ATP-binding cassette sub-family member B

The ABC sub-family B is composed of four full-transporters and seven half-transporters implicated in resistance to drugs. They all have cytoplasmic carboxy- and amino-terminal ends [166].

- **ABCB1**

ABCB1, or multidrug resistance p-glycoprotein, is a full-transporter whose gene is located on the chromosome 7q21.12 that counts 28 exons and encodes for 1280 amino acids [180]. It is mostly expressed at the BBB, in kidneys, at the apical membranes of the hepatocytes and of the enterocytes [170]. Its localization prevents the body from drug toxicity but can also induce a drug resistance, reducing the intracellular concentrations. ABCB1 is a transmembrane glycoprotein with a molecular mass of 170 kDa that is composed of two TMD composed of six MSD followed by a NBD as represented on Figure 1.12 [93, 181]. The

most relevant coding SNPs affecting *ABCB1* are detailed in Table 1.9. The relevant non-coding SNPs can be found in the Chapter VII – Annexes (Table 7.4).

SNPs	Frequency	<i>In vitro and/or in vivo effect</i>	
rs2229109 (1) (c.1199G>A, p.Ser400Asn)	AFR: 0.006 EAS: 0.000 SAS: 0.015 EUR: 0.038	Variant has no effect on tacrolimus intracellular accumulation in K562 or in HEK293 cell model [182] In K562 model, variant protein transport more efficiently imatinib, nilotinib and dasatinib ↓ their antiproliferative effects [183] No effect on darunavir transport [184]	
rs1128503 (2) (c.1236C>T, p.412Gly=)	AFR: 0.813 EAS: 0.375 SAS: 0.395 EUR: 0.564	Females with variant are 2.5-fold more likely to be irresponsive to antiepileptic drugs compared to WT [185] Protective role against severe cytolysis [186] Associated with advanced adverse effects in treatment of non-small cell lung cancer with osimertinib [187]	Chronic myelogenous leukemia ↑ anti-proliferative effect and accumulation of imatinib in 1236T-2677T-3435T variant haplotype vs WT [188] Haplotype in the donor associated with graft loss [189] Haplotype associated with 5-fold chance of requiring high methadone dose [190] No effect on darunavir transport [184]
rs2032582 (3) (c.2677G>T/A, p.Ala893Ser/Thr)	AFR: 0.02-0 EAS: 0.37-0.06 SAS: 0.59-0.05 EUR: 0.41-0.02	Associated with ↑ risk of ATV-induced liver injury [191] Protective role against severe cytolysis [186]	GC haplotype : ATV less efficient to ↓ LDL-C in women [192]
rs1045642 (4) (c.3435C>T, p.1145Ile=)	AFR: 0.808 EAS: 0.605 SAS: 0.397 EUR: 0.461	Associated in a meta-analysis with ↑ risk of ischemic and coronary heart disease [193-195] Donor expressing 3435TT associated with cyclosporine/calcineurin inhibitor allograft nephrotoxicity [196-199] Associated with bleeding complications in patients treated with apixaban and rivaroxaban [200]	

A

OUT

IN

TMD1

TMD2

NBD1

NBD2

NH₂

COOH

(1)

(2)

(3)

(4)

B

OUT

IN

ii. ATP-binding cassette sub-family member C

50

that is developing when *ABCC2* is defective [164, 165]. Except *ABCC13* which is a pseudogene, all *ABCC* transporters are also called multidrug resistance transporters as they detoxify the cells from increased substances accumulation. MRP1 to MRP9 are the proteins encoded by *ABCC1* to *ABCC6* and *ABCC10* to *ABCC12*. Four of the MRPs (MRP4, MRP5, MRP8 and MRP9) are full-transporters whereas the five others (MRP1, MRP2, MRP3, MRP6 and MRP7) also are full-transporters but have an extra TMD [88].

- ***ABCC1***

ABCC1 encodes for the multidrug-resistant protein (MRP1) that is ubiquitously expressed in human tissues but less importantly on the basolateral membranes of the hepatocytes and in the pancreatic tissue [171]. This particularity allows the protein to efflux a wide variety of substrates out of the cells such as organic anions (GSH and the pro-inflammatory cysteinyl leukotriene C₄), peptides for antigen presentation or xenobiotics, including drugs [203]. This efflux transporter is a heavy protein of 190kDa composed of 1531 amino acids encoded by 31 exons localized on the chromosome 16p43.11 that can be affected by many intronic and exonic polymorphisms, as described in Table 1.10 [204]. The relevant non-coding SNPs can be found in the Chapter VII – Annexes (Table 7.5). *ABCC1* expression has been correlated to *ABCC4* expression in our study reported in Chapter IV.

SNPs	Frequency	<i>In vitro</i> and/or <i>in vivo</i> effect
rs246221 (1) (c.825T>C, p.275Val=)	AFR: 0.649 EAS: 0.416 SAS: 0.205 EUR: 0.301	Associated with symptomatic decrease of left ventricular ejection fraction >10% and cardiac failure grade ≥3 [205] Associated with febrile neutropenia in a retrospective study on 994 patients [206] SNP not associated with overall survival in patients with acute myeloid leukemia [207]
rs60782127 (2) (c.1299G>T, p.Arg233Ser)	AFR: 0.002 EAS: 0.000 SAS: 0.003 EUR: 0.013	2-fold ↓ of endogenous substrates transport (leukotriene C ₄ and œstrone sulphate) and 2.1-fold ↑ resistance to doxorubicin [208]
rs45511401 (3) (c.2012G>T, p.Gly671Val)	AFR: 0.011 EAS: 0.000 SAS: 0.016 EUR: 0.056	<i>In vitro</i> , counteraction of SLCO2B1 influx and increase <i>ABCC1</i> activity with SNP [156] <i>In vivo</i> , no association with LDL-C reduction [209], no effect on ATV clearance [51]. ↑ statins LDL-C reduction in 114 adults with familial hypercholesterolemia [210]

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		Deleterious to folate metabolism, no change of the variant on polarity but ↑ the hydrophobicity [211] ↑ risk of chemotherapy-induced cardiotoxicity on a study lead on 1023 patients [212] Associated with febrile neutropenia in a retrospective study on 994 patients [206] Not associated with telatinib PK [213]
rs35529209 (4) (c.2965G>A, p.Ala989Thr)	AFR: 0.000 EAS: 0.000 SAS: 0.001 EUR: 0.001	↓ GSH-dependent inhibitory effect and resistance to vincristine [214]
rs13337489 (5) (c.3140G>C, p. Cys1047Ser)	AFR: 0.051 EAS: 0.000 SAS: 0.000 EUR: 0.000	↓ GSH-dependent inhibitory effect and resistance to epotosside and vincristine [214]
rs2230671 (6) (c.4002G>A, p.1334Ser=)	AFR: 0.098 EAS: 0.117 SAS: 0.169 EUR: 0.280	Associated with ↓ response to citalopram treatment in major depressive disorder [215] SNP has no impact on overall survival in patients with acute myeloid leukemia [207]

Table 1.10. Relevant coding SNPs in ABCC1. Each SNP is identified by rs number and located in both the mRNA and in the protein. The frequency data are issued from gnomAD v2.1.1 and Ensembl [139-142]. AFR: African, EAS: East Asian, SAS: South Asian, EUR: European and AMR: American.

ABCC1 is formed of a TMD, constituted of five MSD, followed by two TMD containing six MSD and two NBD. The C-terminal being cytosolic and the N-terminal extracytosolic [93]. The six exonic SNPs are localized on Figure 1.13A. In the next section studying the implication of the SNPs in ATV PK modulation and SRM, only the variant rs45511401 with the number (3) on Figure 1.13A, will be further investigated. The non-synonymous SNP is localized in the first NBD. It is however not in one of the critical motifs of the domain but rather between the A-loop and the Walker A motif of the protein [216, 217].

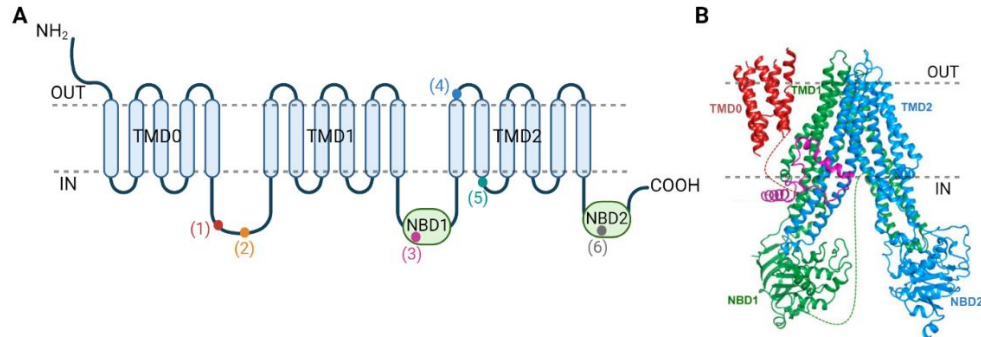


Figure 1.13. ABCC1 topology model (A) and tertiary structure (B). (A) Topology structure including the relevant coding SNPs indicated by numbers referring to Table 1.10. (B) The tertiary structure in inward conformation is adapted from Johnson *et al* [218].

ABCC1 transports GSH and GSH conjugates. These metabolites serve also as co-transported molecules. For instance, the efficient transport of vincristine and mitoxantrone requires GSH and transport of etoposide glucuronide or estrone sulfate is highly increased in presence of GSH. Verapamil and apigenin, although not transported by ABCC1, significantly enhance GSH transport out of the cells. By contrast, some substrates such as organic anions or methotrexate are GSH-independent [88].

- **ABCC2**

ABCC2 encodes for the multidrug resistance protein 2 (MRP2) but unlike other ABCC members, it is mostly found at the apical membranes of polarized hepatocytes, kidney cells and enterocytes (duodenum and intestine) [172]. The gene encodes 1545 amino acids distributed in 32 exons localized on the chromosome 10q24.2 [219]. As for ABCC1, ABCC2 is a heavy protein of 190kDa composed of three TMD and two NBD (Figure 1.14). The most relevant SNPs and their clinical associations are depicted in Table 1.11. The relevant non-coding SNPs can be found in the Chapter VII – Annexes (Table 7.6).

SNPs	Frequency	<i>In vitro</i> and/or <i>in vivo</i> effect
rs2273697 (1) (c.1249G>A, p.Val417Ile)	AFR: 0.183 EAS: 0.088 SAS: 0.278 EUR: 0.197	Reduced ABCC2 placental expression [220] Variant associated with ↑ valproic acid blood concentrations in 187 epileptic patients [221]

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		SNP associated with ↓ mycophenolic acid exposure [222] Significantly ↓ tacrolimus concentrations pre-dose and 2h post-dose by 1.41- and 1.59-fold, respectively [223] Variant ↑ by 3.6-fold the risk of severe cytotoxicity in 63 patients with advanced soft tissue sarcoma [186]	
rs3740066 (3) (c.3972C>T, p.Ile1324=)	AFR: 0.259 EAS: 0.230 SAS: 0.325 EUR: 0.370	Not associated with changes in <i>ABCC2</i> placental expression [220] No association with change in valproic acid blood concentrations on 187 epileptic patients [221] Significantly ↑ tacrolimus concentrations pre-dose and 2h post-dose by 1.33- and 1.47-fold, respectively [223]	
rs17222723 (2) (c.3563T>A, p.Val1188Glu)	AFR: 0.060 EAS: 0.000 SAS: 0.019 EUR: 0.057	Protective role against severe cytotoxicity [186]	
rs8187710 (4) (c.4544G>A, p.Cys1515Tyr)	AFR: 0.160 EAS: 0.000 SAS: 0.019 EUR: 0.057	Homo- and heterozygous carriers associated with 4-fold ↑ risk of congestive heart failure after hematopoietic cell transplantation [227] Variant cause protein ATP activity impairment and ↑ intracellular accumulation of lopinavir, calcein and carboxyfluorescein diacetate compared to WT [228] Associated with ↑ lopinavir accumulation in peripheral blood mononuclear cells of 53 HIV-treated patients [229] Variant associated with 2.19-fold ↑ risk of cardiotoxicity in a meta-analysis [212]	Haplotype associated with 2.3 higher odds of acute anthracycline-induced cardiotoxicity [224, 225] Significant association of the haplotype and susceptibility to nonalcoholic fatty liver disease [226]

Table 1.11. Relevant coding SNPs in *ABCC2*. Each SNP is identified by rs number and located in both the mRNA and in the protein. The frequency data are issued from gnomAD v2.1.1 and Ensembl [139-142]. AFR: African, EAS: East Asian, SAS: South Asian, EUR: European and AMR: American.

The Figure 1.14A represents the topology structure of *ABCC2* comprising the four coding SNPs presented in Table 1.11. Three SNPs will be highlighted in the section “Sources of variability influencing atorvastatin pharmacokinetics and muscular toxicity”. The rs717620

is however not represented as it is localized in the 3' untranslated region of the gene. The two others are rs2273697 and rs3740066, identified by the numbers (1) and (3) on the protein. rs2273697 (1) is non-synonymous but does not modify the hydrophobicity or the interaction inside the cytoplasmic topological domain. rs3740066 (3) is synonymous and localized between the A loop and the Walker A motif of the second NBD [216, 230].

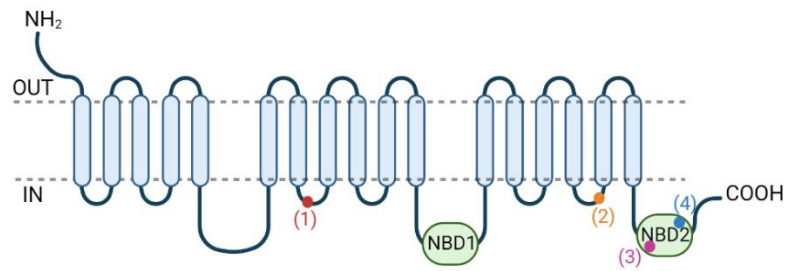


Figure 1.14. ABCC2 topology model. Topology structure including the relevant coding SNPs indicated by numbers referring to Table 1.11.

ABCC2 impairment, resulting from multiple inheritable genetic variations in *ABCC2*, has been associated with the Dubin-Johnson syndrome which is a relatively rare autosomal recessive liver disorder characterized by chronic jaundice, conjugated hyperbilirubinemia and dark pigment deposition in the liver, without other features of hepatobiliary disease [231, 232].

- ***ABCC4***

ABCC4, also called multidrug resistance protein 4 (MRP4), it is found in various tissues including the gastrointestinal tract, endocrine and male tissues and skeletal muscle tissue. Depending on the nature of the tissue, it is localized at the apical or basolateral side of membranes [173]. MRP4 is the smallest ABCC transporter that contains 1325 amino acids encoded by 31 exons localized on the chromosome 13q12.1 [180]. The protein is a full transporter of 170kDa possessing the same topology as ABCB1. SNPs in *ABCC4* have been explored for their clinical implication, as represented in Table 1.12. The relevant non-coding SNPs can be found in the Chapter VII – Annexes (Table 7.7).

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SNPs	Frequency	<i>In vitro</i> and/or <i>in vivo</i> effect
rs4148460 (1) (c.511T>G, p.Cys171Gly)	AFR: 0.000 EAS: 0.000 SAS: 0.000 EUR: 0.000	↑ monomethylarsonous acid transport <i>in vitro</i> [233]
rs11568658 (2) (c.559G>T, p.Gly187Trp)	AFR: 0.004 EAS: 0.124 SAS: 0.061 EUR: 0.022	↓ EC ₅₀ of SN-38 (anticancer drug) by 1.84-fold in variant cell carriers compared to WT, <i>in vitro</i> [234] ↓ EC ₅₀ of azathioprine against cells expressing the variant [235]
rs2274407 (3) (c.912G>T, p.Lys304Asn)	AFR: 0.170 EAS: 0.214 SAS: 0.135 EUR: 0.066	↓ dimethylarsinic acid and monomethylarsonous acid transport <i>in vitro</i> [233] Variant associated with ↑ thrombocytopenia and ↓ event-free survival in childhood acute lymphoblastic leukemia [236]
rs2274405 (4) (c.969A>G, p.323Ser=)	AFR: 0.688 EAS: 0.555 SAS: 0.643 EUR: 0.664	↑ response in 120 esophageal cancer patients to platinum-based therapy [237]
rs753414892 (5) (c.1667A>G, p.Tyr556Cys)	AFR: 0.000 EAS: 0.000 SAS: 0.000 EUR: 0.000	↑ dimethylarsinic acid and prostaglandin E ₂ transport <i>in vitro</i> [233]
rs3765534 (6) (c.2269G>A, p.Glu757Lys)	AFR: 0.003 EAS: 0.108 SAS: 0.050 EUR: 0.011	↑ 6-thioguanine nucleotide and ↓ white blood cell levels with variant [238] Significant ↓ of protein expression at the cell membrane [239] ↓ EC ₅₀ of azathioprine against cells expressing the variant [235]

Table 1.12. Relevant coding SNPs in *ABCC4*. Each SNP is identified by rs number and located in both the mRNA and in the protein. The frequency data are issued from gnomAD v2.1.1 and Ensembl [139-142]. AFR: African, EAS: East Asian, SAS: South Asian, EUR: European and AMR: American.

Six of the most relevant SNPs in *ABCC4* are coding and represented on the topological structure of the protein. The SNP rs3742106 studied in the section pharmacogenetics could not be represented on Figure 1.15A as it is localized in the 3' untranslated region of the gene [142, 240].

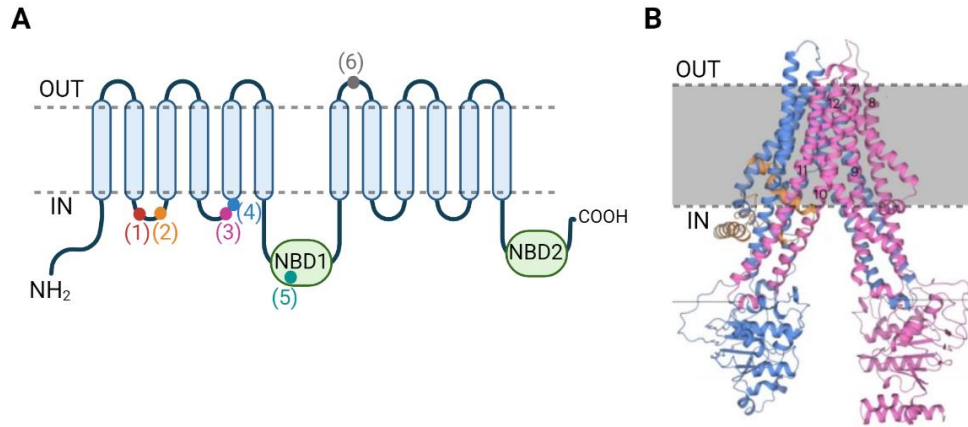


Figure 1.15. ABCC4 topology model (A) and tertiary structure (B). (A) Topology of MRP4 protein including the relevant coding SNPs indicated by numbers referring to Table 1.12. (B) The tertiary structure is adapted from Chen et al [241].

ABCC4 has been identified as a key factor of bad cancer prognosis. It was demonstrated that high levels of expression of the transporter promotes the proliferation of pancreatic ductal adenocarcinoma cells, the cancer being more aggressive [242]. Doxorubicin which is an anticancer drug used to treat a wide range of tumors is being transported by ABCC4. In osteosarcoma cells overexpressing that protein induced a resistance to the anticancer drug [243]. Blocking ABCC4 with anti-androgen treatment in castration resistant prostate cancer might result in a better survival rate [244]. All in all, ABCC4 seems to play a major role in the onset of various cancers as well as in their treatment.

iii. ATP-binding cassette sub-family member G

This family is coding for five “reverse” half-transporters. Half-transporter as they only contain the second TMD and second NBD. And reverse because their N-terminal ends are cytoplasmic [164]. Disorders induced by ABCG1, ABCG5 and ABCG8 transporters of this sub-family are atherosclerosis, type II diabetes and sitosterolemia. ABCG1, ABCG2 and ABCG4 have been linked to the development of Alzheimer’s disease [245]. Those proteins mostly transport phospholipids and sterols [179].

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- **ABCG2**

ABCG2, or breast cancer resistance protein (BCRP), is a half-transporter located on the chromosome 4q22 counting 16 exons. The protein contains 655 amino acids and the molecular weight of ABCG2 monomer is 70kDa [246]. It is localized all along the gastrointestinal tract, in the liver, at the BBB, and in the skeletal muscle tissue [176]. Most of the relevant SNPs are intronic, as represented in Table 1.13. The relevant non-coding SNPs can be found in the Chapter VII – Annexes (Table 7.8).

SNPs	Frequency	<i>In vitro and/or in vivo effect</i>
rs2231137 (1) (c.34G>A, p.Val12Met)	AFR: 0.064 EAS: 0.271 SAS: 0.141 EUR: 0.034	Associated with advanced adverse effects in treatment of non-small cell lung cancer with osimertinib [187] Associated with poor response to imatinib mesylate therapy in chronic myeloid leukemia patients
rs2231142 (2) (c.421C>A, p.Gln141Lys)	AFR: 0.027 EAS: 0.307 SAS: 0.093 EUR: 0.104	In homozygous: ↑ ATV AUC _{0-∞} and C _{max} [129, 247] Variant ↑ uric acid levels and the risk of gout [248] Gefitinib toxicity in heterozygous carriers on 124 patients with non-small cell lung cancer [249] Variant associated in a meta-analysis with significant ↑ rate of major molecular response with imatinib mesylate treatment in chronic myeloid leukemia [250] Heterozygotes associated with ↓ risk of psoriasis [251] Significantly associated with lamotrigine concentrations [252]

Table 1.13. Relevant coding SNPs in ABCG2. Each SNP is identified by rs number and located in both the mRNA and in the protein. The frequency data are issued from gnomAD v2.1.1 and Ensembl [139-142]. AFR: African, EAS: East Asian, SAS: South Asian, EUR: European and AMR: American.

Alike all ABCG sub-family members, ABCG2 is composed of one TMD domain preceded of one NBD and both N- and C-terminal ends are cytoplasmic (Figure 1.16A). The longer extracellular loop between the 5th and the 6th MSD of the protein is visible on the tertiary structure (Figure 1.16B), represented on top of the structure. The two coding SNPs rs2231137 (1) and rs2231142 (2) are represented on the topological structure of ABCG2, on Figure 1.16A. Whereas rs2231137 (1) is located ahead of the Walker motifs of the NBD, rs2231142 (2) is located between the Walker A motif and the ABC signature of the NBD

[177]. In the wild-type condition, the glycine creates a hydrogen bond with the parallel α -helix. The non-synonymous SNP replaces it with a lysine that can also create a hydrogen bond but is a bigger amino acid which could supposedly modify the orientation of the α -helix [253].

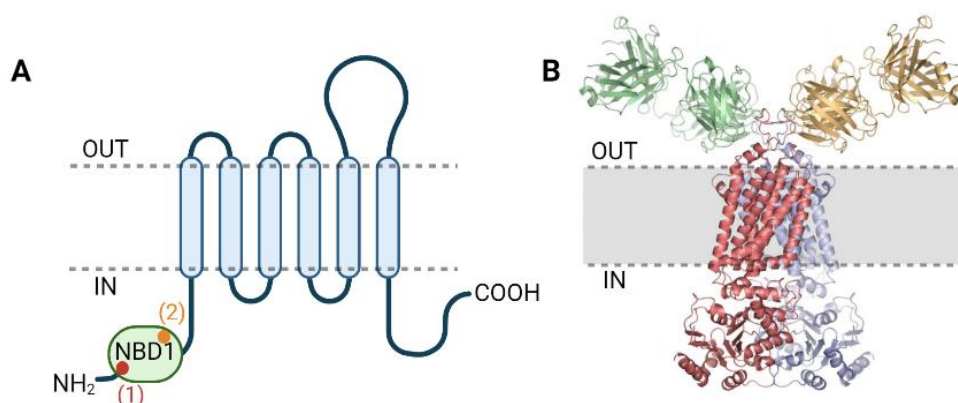


Figure 1.16. ABCG2 topology model (A) and tertiary structure (B). (A) Topology of the protein including the relevant coding SNPs indicated by numbers referring to Table 1.13. (B) The tertiary structure in outward conformation is adapted from Taylor *et al* [253].

ABCG2 is also called BCRP (breast cancer resistance protein) because of its implication in breast cancer but also in leukemia and acute myeloid leukemia cancer resistance. It has indeed been demonstrated that high level of expression of the protein is implicated in poor cancer prognosis as the efflux pump will decrease the tissue penetration and concentration of the anticancer drugs [254].

c) Transporters-metabolism interplay

ADME is partially ruled by influx and efflux transporters (Figure 1.17) being expressed in all body compartments. As an example, OATP1B1 and OATP1B3, mostly expressed at the sinusoidal membranes of the hepatocytes, will regulate the pharmacologically active ATV acid that reaches the hepatocytes to exert its therapeutic activity but also to be biotransformed. It will be metabolized in its corresponding lactone form and both are further metabolized into OH-metabolites by the cytochrome P450 (CYP) 3A isoenzymes

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(Figure 1.17). The main metabolite, 2-OH-ATV (ortho-), is pharmacologically active and significantly contributes to the inhibitory activity on HMG-CoA reductase but, little is known about its potential involvement in myotoxicity [81]. Efflux transporters also present at the sinusoidal hepatocyte membranes will limit the access of the drug and thus the metabolization, whereas those present at the canalicular border will help excrete the drug or its metabolites in the bile ducts. ATV acid is lipophilic, however less importantly than simvastatin or fluvastatin also included in that category [255]. The lipophilicity of the ATV lactone form is 3.8 more lipophilic than its acid form according to their Log P partition coefficient [255, 256].

To get into myocytes, where toxicity occurs, ATV (and possibly its metabolites) undergoes passive diffusion but also active and facilitated transport mechanisms. The activity of transporters is thus assumed to modulate drug concentrations directly within myocytes through both drug efflux and influx. Interestingly, *ABCC1*, *ABCC2*, *ABCC4*, *ABCG2*, *SLCO2B1* and *SLCO1B1* are expressed in the skeletal muscle and might play an important role in protecting and sensitizing myocytes from ATV toxicity [108, 171-173]. On the other hand, some of these transporters are expressed in the liver where the drug acts on cholesterol synthesis. Whereas OATP transporters (*SLCO1B1*, *1B3* ad *2B1*) drive the influx of drug within the hepatocyte and by such, increases the fraction of the drug reaching its target, *ABC* transporters will either limit the access to the liver at the basolateral pole (*ABCC1* and *ABCC4*) or increase its excretion when expressed apically (*ABCB1* and *ABCC2*) [257]. Depending on the localization of the different transporters but also the likelihood of compensation for a potential loss of activity, functional genetic defects would not have the same impact on systemic versus local muscle drug exposure. For instance, whereas most transporters are substantially expressed in the liver, only a few of them are expressed at a significant level in the muscle tissue. The net consequence of this consideration is that, even if a change in the activity does not show any association with the systemic exposure because other transporters might compensate the decreased activity in excretory organs, it does not mean that it cannot impact on the susceptibility of muscle toxicity by directly modulating the local exposure where other transporters are not (or less) expressed.

Chemically, ATV is composed of a dihydroxy heptanoic side chain where a lactone group can be added by phase-II glucuronosyltransferases (*UGT1A3* and *UGT1A1*) or through spontaneous deconjugation. Inversely, ATV lactone can be hydrolyzed back to the active acid form by esterases such as paraoxonases (*PON1* and *PON3*) [81, 258-260]. As stated above, in the liver, ATV biotransformation is driven by CYP enzymes into 2 (ortho) and 4 (para)-OH metabolites. It has been reported that more than 85% of the oxidative metabolism is catalyzed by CYP3A4, whereas it is estimated that CYP3A5 contributes to less than 15% of the phase-I metabolism [261]. 98% of ATV is bound to plasma proteins but, given its lipophilicity, it is characterized by a large volume of distribution (381L) [262, 263], indicating that a high proportion of ATV is found in extravascular compartments [264]. ATV is mainly eliminated through biliary excretion (70%) after hydroxylation of its lactone form [80, 265]. Despite its inability to inhibit the HMG-CoA reductase, the lactone has been shown to be up to 14-fold more potent than the acid form to induce myotoxicities *in vitro*. This discovery is based on the decreased viability of the myotubes in presence of the lactone form compared to the acid one [59]. This phenomenon is supposed to be due to the 2.7-fold more lipophilic property of the lactone form when compared to its acid counterpart, allowing the molecule to cross the skeletal muscle cells more easily where it is then converted back into the acid form that is myotoxic [266]. The manifestation of myalgia might thus be indirectly triggered by the lactone form but imputable to ATV acid intramuscular accumulation due to this interconversion. Because of its lower lipophilicity compared to lactone, ATV acid is further modulated by influx and efflux transporters expressed on the cell membranes. Their expression levels and/or the presence of SNPs could therefore influence the ATV acid intracellular content hence modulating the individual risk to develop muscle symptoms. To a further extent, the nature of the statin also defines the development of adverse events, the lipophilic statins (e.g. fluvastatin) being more toxic than the hydrophilic statins (e.g. pravastatin) [267].

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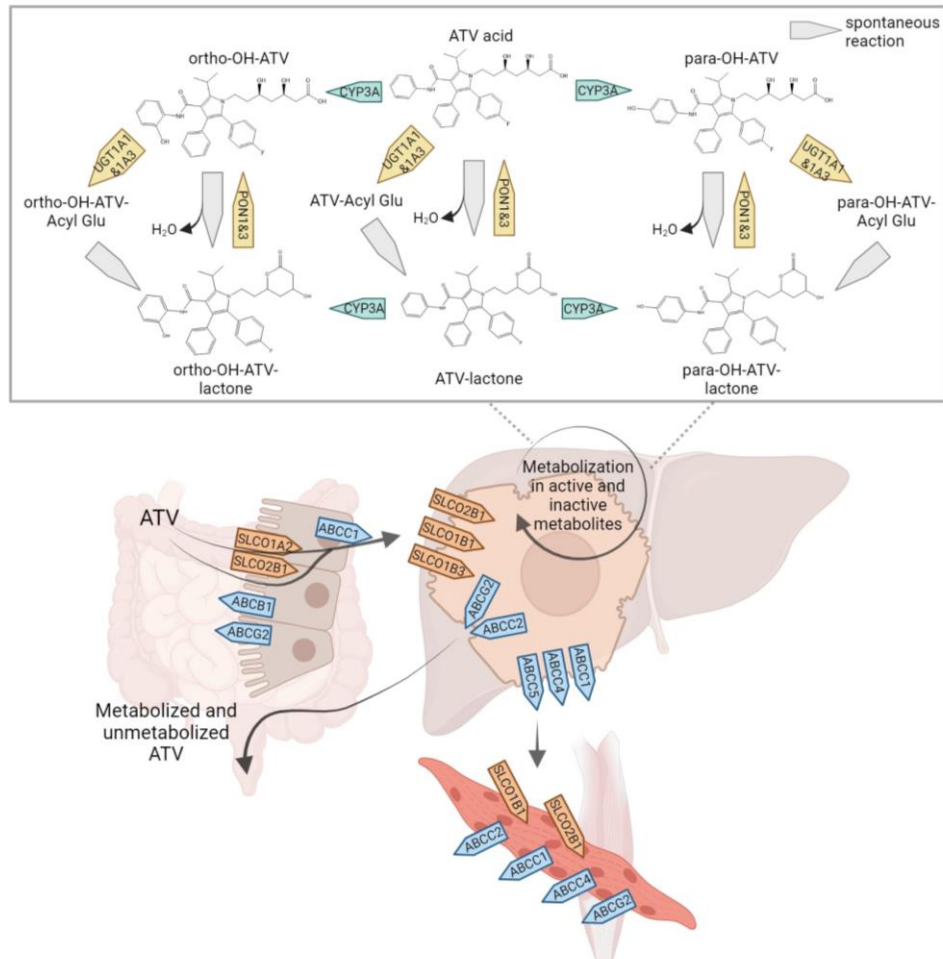


Figure 1.17. Overview of ATV transportation and metabolism in the different body compartments. Once ATV is ingested, it is absorbed passively or via influx transporters (orange indicators). At this stage, the efflux transporters (blue indicators) present at the apical membrane of the enterocytes will limit its absorption. ATV then enters the hepatocytes via influx transporters where it is being metabolized into its active and inactive metabolites. They subsequently are either expelled via bile ducts or reach other body compartments such as the skeletal muscle cells. Created with BioRender.com.

3. Sources of variability influencing atorvastatin pharmacokinetics and muscular toxicity

A. Pharmacogenetics

a) Drug transporters

Genes coding for transporter proteins are highly polymorphic and their genetic sequences can be affected by SNPs. Depending on the functionality of the SNP, the transporter activity, transporter localization as well as its affinity for ATV, SNPs can be associated with either increased/decreased or no change in ATV systemic and/or local exposure with potential impact on the response to the drug (see Table 1.14).

i. Efflux transporters

- **ABCB1**

ABCB1, formerly referred as P-glycoprotein (P-gp) or multidrug resistance 1 (MDR1), mainly protects the organism from an excessive substrate accumulation due to its ubiquitous expression. Depending on its location, ABCB1 restricts the drug bioavailability when expressed in the intestinal lumen, limits the access to potential toxic substances to some fragile tissues (e.g. BBB) or controls systemic drug exposure by eliminating substrates via the corporeal fluids through its expression in excretory organs [170]. An altered transport activity can thus modulate drug disposition and consequently modify the drug efficiency and/or toxicity. Several pharmacogenetic studies have attempted to determine whether *ABCB1* variants impact on ATV PK. Kajinami *et al.* studied the SNP rs2032582 (c.2677G>T/A, p.Ala893Ser/Thr) and rs1045642 (c.3435C>T, p.Ile1145=), both in strong linkage disequilibrium, in a cohort of 344 hypercholesterolaemic patients treated with low dose ATV (10mg per day). They showed that women carrying the wild-type haplotype (2677G-3435C) were characterized by a lower LDL-C reduction when compared to the 2677T/3435T variant haplotype carriers resulting from lower circulating ATV concentrations. This observation was not replicated in men. The higher ATV exposure and the consequent better LDL-C decrease in variant haplotype carriers might be explained by a lower efflux pump activity when the SNP is present [192]. Also, when considering the rs1045642 SNP only,

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women homozygous wild-type (3435CC) experienced significantly lower LDL-C reduction but increased HDL-C levels indicating that the 3435C>T SNP might be responsible for the haplotype association [192]. Those results were later supported by the research team of Kadam *et al.* who also showed a greater reduction of LDL-C blood levels in 3435T variant carriers when compared to the wild-type *ABCB1* expressors, but they did not assess ATV PK [268]. In another PK study performed on 60 male volunteers taking one single 80mg ATV dose, it was shown that the variant allele (3435T) was associated with a significant increase of C_{max} compared to homozygous wild-type allele carriers (3435CC) [269]. To our knowledge, none of the studies reported to date have evidenced that this increased systemic exposure in variant allele carriers translates into an increased risk of developing SRM.

Another less commonly studied intronic SNP rs2373588 (g.194405C>T) in the efflux transporter *ABCB1* has been investigated for its impact on ATV toxicity in a large case-control study including 1102 acute ischemic stroke patients receiving 80mg ATV/day *de novo* and experiencing or not myopathy, as defined by CK levels >10 times the ULN. In that study, it was reported that the *ABCB1* rs2373588 194405T variant allele was more frequent in the cases than in the controls but, again, no PK endpoint was assessed [270]. To our knowledge, no other study has evaluated the robustness of this association.

- ***ABCC1***

The ATP-binding cassette family member 1 (*ABCC1*) is ubiquitously expressed in the human body with some tissues expressing more (e.g. kidneys, gonads) or less (e.g. liver, endocrine tissues) importantly the protein [171]. In polarized cells, the protein is localized on the basolateral plasma membrane [271]. Its substrates are thus expelled in the interstitial spaces instead of extracorporeal fluids (Figure 1.17).

In 2017, Behdad *et al.* showed, in a cohort of 179 Iranian patients with primary hypercholesterolemia, no significant association between the *ABCC1* rs45511401 (c.2012G>T, p.Gly671Val) polymorphism and LDL-C reduction after four weeks of treatment with different ATV dosages [209]. This SNP was also evaluated for its effect on ATV PK in a population PK study investigating the effect of SNP on ATV apparent clearance and clinical response in 70 ATV treated patients. No effect of the c.2012G>T was observed on ATV

clearance and, therefore, the association with clinical response endpoints was not tested [51]. In a recent *in vitro* study using HEK293 recombinant cell lines over-expressing *ABCC1* alone or in combination with *SLCO2B1*, it was observed that increased ATV intracellular accumulation driven by *SLCO2B1* influx was partially counteracted by *ABCC1* efflux and that the c.2012G>T SNP in *ABCC1* was associated with increased *ABCC1* efflux activity towards ATV [156], indicating a potential importance of this SNP to modulate the activity of *ABCC1* and protect against excessive drug accumulation within the cell. Future more powerful studies are needed to conclude about the clinical relevance of this functional effect and the potential consequence of this *in vitro* observation on SRM occurrence.

- ***ABCC2***

The ATP-binding cassette family member 2 (*ABCC2*) efflux protein is located on the apical plasma membrane of the hepatocytes, the BBB, the kidneys and the intestinal tract (Figure 1.17) [172, 272]. It transports endogenous substrates like taurolithocholate sulfate and also exogenous substances such as statins and HIV drugs [174, 273]. In 2013, Becker *et al.* analyzed, in a cohort of 225 ATV users (10mg, 20mg and 40mg daily), whether the common SNPs rs717620 (c.-24C>T), rs2273697 (c.1249G>A, p.Val417Ile) and rs3740066 (c.3972C>T, p.Ile1324=) in the *ABCC2* gene were associated with a dose decrease or switch to another cholesterol-lowering drug. They highlighted that H12 haplotype (–24T/1249G/3972T) carriers were at higher risk for these events compared to carriers of the H2 haplotype (–24C/1249A/3972C). Although non-significant for ATV users, this association might indirectly reflect that patients have developed adverse muscular reactions needing dosage or therapy adjustments [274]. The fact that the difference was not significant might be due to the imprecise outcome definition as the switch or dose decrease might have multiple causes other than drug side-effect. As, to our knowledge, the impact of *ABCC2* SNP on ATV PK or *in vitro* transport has not yet been assessed, it is difficult to conclude on the relevance of this observation and further studies are needed.

- ***ABCC4***

Multidrug resistance-associated protein 4 (MRP4), is also a member of the ATP-binding cassette family transporting ATV out of the cells [174]. It is mainly found at the basolateral membrane of the hepatocytes, the gastrointestinal tract and the male and female sexual

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organs (Figure 1.17) [173]. Very few research groups investigated the impact of SNP in *ABCC4* on ATV PK. However, Jiang *et al.* recently highlighted in a cohort of 212 out- and in-patients suffering from chronic kidney disease (CKD) treated with 20mg of ATV/day for 6 weeks, through univariate and multivariate analysis, that carriers of the variant rs3742106, located in the 3'UTR (c.*38T>G), was associated with reduced ATV and its metabolites plasma concentrations compared to the wild-type carriers. This would indicate a decreased efflux transporter's activity. Indeed, as *ABCC4* is expressed at the basolateral membrane of cells, a decreased activity would thus theoretically lead to a reduced recirculation of the drug and thus lower plasma levels. However, as the study includes CKD patients only, it is difficult to conclude whether these results can be extended to other less specific patient populations [275].

- ***ABCG2***

ABCG2, also known as breast cancer resistant protein (BCRP), is one of the efflux transporters that is the most studied for its impact on ATV PK modulation mainly due to its location. It is indeed expressed at the apical membrane of the hepatocytes, the enterocytes, the renal proximal tubule cells and at the BBB (Figure 1.17) [176, 276]. Among *ABCG2* SNP, the rs2231142 (c.421C>A, p.Gln141Lys) has received much attention. In a cohort of 32 healthy individual receiving a single 20-mg ATV oral dose, Keskitalo *et al.* highlighted that the area under the curve ($AUC_{0-\infty}$) in patients expressing the variant genotype (c.421AA) was 72% greater than in those with the wild-type genotype (c.421CC) [247]. The observation that the $AUC_{0-\infty}$ of ATV is affected by the *ABCG2* genotype, with no effect on its elimination half-life ($t_{1/2}$), suggests that *ABCG2* function mainly affects the ATV absorption or distribution with limited influence on elimination rate [247]. Going in the same direction, Birmingham *et al.* carried out a study on 93 Chinese, Japanese and Caucasian volunteers receiving 40mg of ATV per day. They also highlighted an increased ATV $AUC_{0-\infty}$ and C_{max} in *ABCG2* c.421AA compared to c.421CC carriers [129]. As the rs22431142 SNP is more frequent in Asians, these observations might explain why ethnicity plays a modulating role in ATV PK. However, the recent study of Lee *et al.* failed to replicate this observation in a two-period crossover study involving 47 statin-naïve Japanese patients receiving an oral micro dose (100µg) of ATV followed by a low therapeutic dose (10mg). Contrarily to the other studies, it was

observed that, even if the differences did not reach statistical significance, ATV exposure in patients with the *ABCG2* c.421AA variant genotype tended to be reduced when compared to patient carriers of the c.421CC or c.421CA genotypes either after 100µg or 10mg ATV dosing [277]. Regarding the ATV efficacy, in a study enrolling 127 hypercholesterolaemic patients treated with 10mg of ATV for 4 weeks, Prado *et al.* did not find any change in the LDL-C response to ATV between the c.421CC carriers and the c.421AC or AA carriers but no PK data were reported [278]. Interestingly, a case-control study comparing data from 60 patients who experienced ATV dose-related side effects with 90 matched control patients without side-effect, showed that patients with *ABCG2* 421CA or AA genotypes had 2.9 times greater odds of developing ATV side-effects compared to patients with *ABCG2* 421CC genotype [279]. All in all, because the impact of the SNP rs2231142 (c.421C>A) in *ABCG2* on ATV PK is still unclear, there is no guideline considering the rs2231142 variant regarding the use of ATV [280]. However, taken together, reported associations suggest that the rs2231142 SNP in *ABCG2* is associated with decreased efflux activity towards ATV and impacts on either the drug absorption or distribution with little or no effect on drug excretion. As the SNP is associated with differences in the odds of suffering from side-effects but not with drug efficacy, it might indicate that the transporter impacts more on the local drug accumulation in specific tissues rather than on the systemic/liver drug accumulation because of potential compensatory mechanisms present in the liver. Further mechanistic studies are needed to evaluate the impact of this SNP on ATV muscle intracellular PK.

ii. Influx transporters

- ***SLCO1B1***

OATP1B1 encoded by *SLCO1B1* is involved in the hepatic uptake of the drug and is the influx transporter in the center of all attention when studying ATV PK or pharmacodynamic modulations. OATP1B1 is expressed at the basolateral membranes of hepatocytes only, where it facilitates the hepatic extraction of the drug, thereby favoring its therapeutic effect, but, paradoxically, also its hepatic excretion and thus limiting systemic drug exposure (Figure 1.17). This exclusive location together with the known functional defect associated with the most studied SNP rs4149056 (c.521T>C, p.Val174Ala) probably explains why the associations observed with ATV PK are more clearcut than for other genes/SNPs [99]. The

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missense variant rs4149056 has been largely studied for its impact on statin PK [281]. it has been shown *in vitro* that the c.521T>C SNP is the key SNP that determines the functional polymorphic activity of the transporter and is associated with decreased activity [145]. Turner *et al.* performed a genome-wide association recruiting 590 acute coronary syndrome hospitalized patients treated with high doses of ATV (40 or 80mg/day). The variant 521C minor allele was clearly associated with higher ATV and 2-OH ATV concentrations. By contrast, after adjustment for multiple testing, this was not observed for ATV lactone and for 2-OH ATV lactone [128]. Confirming this observation, two independent research groups demonstrated that both c.521CC variant homozygous and c.521TC heterozygous genotypes were associated with increased ATV AUC and C_{max} compared to the c.521TT wild-type genotype [129, 130]. Indeed, Birmingham *et al.* associated the variant in a mixed cohort of 93 Chinese, Japanese and Caucasian subjects receiving 40mg of ATV once a day with higher exposure to the drug with the effect consistent across ethnic groups [129]. Pasanen *et al.* studied 32 healthy volunteers treated with a single ATV 20mg dose. Variant homozygous (c.521CC) had a 144% ($P > 0.001$) greater AUC_{0-48h} than the homozygous wild-type individuals (c.521TT) and a 61% ($P = 0.049$) greater AUC_{0-48h} than heterozygous (c.521TC) [130]. Surprisingly, Giannakopoulou *et al.* conducted a study on 201 Greek adults treated for a primary hypercholesterolemia with ATV (5-80mg/day) and found that the polymorphism (c.521T>C) had no-significant association with the ATV-lowering response, suggesting that even if the SNP impacts on ATV exposure, it has no or little impact on its therapeutic efficacy. They also investigated two other *SLCO1B1* variants; rs2306283 (c.388A>G, p.Asn130Asp) and rs201149584 (c.411G>A, p.Ser137=) but, again, did not observe any significant impact on the ATV PK [121]. This observation is corroborated by the genome wide association study of Turner *et al.* where they showed that even if the c.521T>C SNP was associated with ATV PK, it was not linked to the efficacy endpoints as assessed by the development of myocardial infarction, ischemic stroke, cardiovascular death or all-cause mortality. By contrast, when considering muscular toxicity endpoints, they observed that *SLCO1B1* rs4149056 increased the risk of muscular complaints and ATV intolerance [128]. This genome wide association study (GWAS) however highlights the implication of single

polymorphisms and, their combination was not considered which might be worth considering in future investigations.

Other studies also demonstrated an increased susceptibility of this variant to develop ATV toxicity. Indeed, Stillemans *et al.* studied the impact of the SNP c.521T>C in *SLCO1B1* and highlighted, based on a population PK model, that ATV clearance was reduced in variant carriers (521C) compared to the wild-type carriers (521T). It was shown that a reduction of ATV clearance below $414.67 \text{ L}\cdot\text{h}^{-1}$ put the patients at risk of experiencing SRM. The results were acquired on 132 PK samples from a cohort of 70 patients taking 10 to 80mg of ATV [51]. A retrospective survey data from 379 genotyped Caucasian subjects highlighted an association between the variant c.521T>C and an ATV discontinuation due to the development of muscle side-effects [131]. Even if few studies failed to associate this *SLCO1B1* SNP with myotoxicity endpoints [121, 128], a meta-analysis combining data extracted from 15 studies consistently showed that 521C heterozygous and homozygous carriers are at up to ~ 2.0 and 4.0 times higher odds of SRM compared to 521TT wild-type genotype [132]. The apparent discrepancy between some studies might potentially be attributed to the different ATV doses administered or the composition of the population. Indeed, the different results indicate that the genetic association between SRM and *SLCO1B1* is mostly observed with high ATV dose and only when the cohort includes enough patients taking ATV. For instance, in a less recent meta-analysis, the rs4149056 was significantly associated with simvastatin but not with ATV SRM. However, in the included studies, fewer participants took ATV relative to simvastatin for which the risk of developing side-effect is higher [282]. Very recently, in a double blinded study including 28 coronary patients with self-perceived SRM treated with 40mg ATV or placebo in a randomized order for 7 to 8 weeks, Lauritzen *et al.* examined whether ATV metabolites exposure in blood reflects the local exposure in the skeletal muscle and whether genetic variants in transporters modulate this relationship. The authors concluded that ATV metabolite levels in skeletal muscle and plasma are strongly correlated, implying that plasma measurements are suitable proxies of ATV exposure in muscle tissue. However, when looking at the data, they showed that the correlation between the sum of ATV acid metabolites in the muscle and in the plasma was characterized by a rho value (spearman correlation) of 0.7 which is

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considered as a moderate to strong correlation (but not very strong as stated in their conclusions). Visually, among the 26 eligible patients for PK analysis, several points were far from the identity line, indicating that the correlation, despite statistically significant, is not verified for all patients. They showed that the *SLCO1B1* 521C carriers had higher median levels of acid and lactone ATV metabolites in the muscle which was probably a consequence of a less efficient liver excretion and higher ATV plasma exposure. This is in line with meta-analysis linking this SNP with SRM susceptibility. However, given the small number of patients included in their study, despite informative, it is difficult to draw any definitive conclusion about the correlation between ATV muscle and blood concentrations or SRM and the impact of other SNPs that could affect the local exposure without impacting systemic ATV concentrations [154]. Recommendations have been established based on studies that associated the *SLCO1B1* phenotype with the development of SRM [280].

- ***SLCO1B3***

Solute carrier organic anion transporter family member 1B3 (*SLCO1B3*) encodes for OATP1B3, an influx transporter mainly localized in the liver but less importantly than *SLCO1B1* which remains the most expressed (Figure 1.17) [105]. Belonging to the same subfamily, their substrate spectrum is similar without being identical. *In vitro*, Schwarz *et al.* showed by transient plasmid expression that, even if rs57585902 (c.439A>G p.Thr147Ala) rs60140950 (c.767G>C p.Gly228Ala) and rs2043949386 (c.1559A>C, p.His520Pro) *SLCO1B3* variant haplotype results in altered expression, substrate specificity and pH-dependent activity, it does not seem to impact ATV transport significantly [148]. Accordingly, Stillemans *et al.* did not find any significant association between the *SLCO1B3* rs4149117 (c.334T>G, p.Ser112Ala) SNP and ATV clearance, self-reported muscle toxicity or LDL-C lowering response in a cohort of 70 ATV users [51]. These results indicate that, even if OATP1B3 transports ATV, the functionality of natural SNP is not strong enough to impact on ATV PK, especially given the fact that OATP1B1 can eventually compensate for OATP3B1 activity alteration.

- ***SLCO2B1***

The solute carrier organic anion transporting polypeptide 2B1 (*SLCO2B1*) is mostly expressed in the hepatocytes at comparable RNA levels than *SLCO1B3* but less importantly than *SLCO1B1*. Interestingly, by contrast to other SLCO carriers transporting ATV, *SLCO2B1* is the only one that has been described as expressed on the membrane of skeletal muscle tissues (Figure 1.17) [108]. It mediates the Na⁽⁺⁾ independent transport of organic anions and endogenous substances such as taurocholate, estrone 3-sulfate and of many xenobiotics [283]. Knauer *et al.* have originally demonstrated that OATP2B1 is expressed on the membrane of human skeletal muscle cells and that its expression increases intracellular accumulation and toxicity of statins at supraphysiological drug exposure [174]. *In vitro*, as explained above, using HEK293 recombinant cell lines over-expressing OATP2B1 alone or in combination with ABCC1, it was observed that ATV intracellular accumulation was boosted by OATP2B1 overexpression over a range of ATV concentrations from 25 to 500nM [156]. Also, in that study, it was shown that this boosting effect on ATV cellular accumulation was decreased by the introduction of the rs12422149 (c.935G>A, p.Arg312Gln) SNP in the *SLCO2B1* coding sequence. Similarly, Yang *et al.* 2020, studied the impact of 14 nonsynonymous variants in *SLCO2B1* towards ATV in a recombinant HEK293 cell system among which the frequent c.935G>A SNP. They showed consistently a slightly decreased ATV accumulation in variant cell lines compared to the wild types [155]. In line, in the above-mentioned study, despite the low number of patients carriers of the variant allele, Lauritzen *et al.* observed non-significant lower muscle concentrations of ATV lactone metabolites in *SLCO2B1* variant carriers [154]. By contrast, Stillemans *et al.* did not find any influence of this SNP on ATV clearance in their population PK model. However, Stillemans *et al.* observed that patients co-medicated with an inhibitor of OATP2B1 had lower clearance values than the rest of the population, suggesting a potential importance of OATP2B1 in ATV PK [51]. Again, further studies are needed to confirm the role of the c.935G > A SNP in *SLCO2B1*, if any, in modulating the susceptibility to develop SRM.

b) Biotransformation enzymes

i. Phase I metabolization enzymes

As explained above, ATV is primarily metabolized by CYP3A4 into less active metabolites, see Figure 1.17. The evidence of variants influencing the ATV PK at the biotransformation step are frequent. Such is the case with the *CYP3A4**22 allele (rs35599367, c.522-191C>T) that has been associated with lower *CYP3A4* hepatic expression and activity and might thus account for differences in ATV PK [284, 285]. In line with that hypothesis, Wang *et al.* evidenced that *CYP3A4**22 carriers required significantly lower doses of statins for optimal lipid control and Klein *et al.* that healthy *CYP3A4**22 carriers showed 35% decreased metabolite to parent compound ratio [284, 286]. This influence of the *CYP3A4**22 allele on ATV PK was further confirmed by Kitzmiller *et al.* who showed that statin (including ATV) dose requirement for optimal lipid control was significantly associated with the *CYP3A4/5* combined genotype grouping [287].

Klein *et al.* also associated *CYP3A4* decreased ATV-2-hydroxylation with another deleterious polymorphism (rs4253728, c.209-1003G>A) in the peroxisome proliferator-activated receptor- α (*PPARA*). The genetic variation induces an increase of the unmetabolized circulating ATV, leading to higher systemic exposure to the drug. This was investigated *in vitro* using 150 samples based on a candidate-gene approach from a human liver bank and the results were validated *in vivo* on a cohort of 56 genotyped volunteers [286]. Since *CYP3A* is regulated by *PPARA* which is itself controlled by microRNAs in the liver, research groups identified genetic variations in the microRNAs mi-21, miR-27b and miR-206 that impact their efficiency to regulate *PPARA*. This phenomenon was associated with the development of SRM in several studies [288-292].

As a member of the P450 superfamily, *CYP3A4* activity is modulated by the P450 oxidoreductase (POR) protein activity which is responsible for the transfer of electron from NADPH to CYP450, thereby promoting ATV metabolism. In a clinical trial using *CYP3A* phenotyping probes, the variant rs1057868 (c.1508C>T, p.Ala503Val) defining the *POR**28 allele has been associated with increased *CYP3A* activity [293]. Ragia *et al.* showed a non-significant tendency of the *POR**28 variant towards an increased reduction of the LDL-C and the total cholesterol after 6 months of treatment of 207 adults with hypercholesterolemia

[294]. This observation is counterintuitive as the *POR*28* is associated with CYP3A increased activity; thus higher ATV metabolism, lower drug exposure and decreased efficacy would be expected. As they did not assess ATV PK in their study, we cannot conclude on the causality of their observation. By contrast, and in agreement with the increased CYP3A activity associated with the *POR*28* allele, Drogari *et al.* showed in a cohort of 123 children and adolescents with familial hypercholesterolemia that, after 6 months of treatment with ATV (10, 20 or 40mg/day), patients homozygous *POR*28/POR*28* had significantly higher total cholesterol levels when compared to heterozygous or wild-type *POR*28* genotypes [295]. Again, no PK assessment was performed, and they did not register the occurrence of muscular side-effects in their study. Consequently, it is not yet known if the *POR*28* impact on PK, if any, is affecting the risk of developing SRM.

It is established that CYP3A5 is not the main phase-I metabolism enzyme implicated in ATV biotransformation but there are indications that the variant *CYP3A5*3* (rs776746, c.219-237A>G) might influence ATV-induced muscle side-effects. Indeed, in a balanced case-control study involving 137 individuals, Wilke *et al.* investigated the impact of *CYP3A5*3* allele on ATV SRM occurrence. They highlighted that adults expressing two copies of this variant allele have a greater likelihood to experience myotoxicities but, no PK data were available to confirm the causative association. The observation is in line with the functionality of the SNP as this intronic variant induces an alternative splicing introducing a stop codon that leads to a loss of *CYP3A5* activity thus lower metabolism and possibly higher SRM occurrence [296, 297].

Although ATV is not supposed to be metabolized by CYP2D6, studies have surprisingly evidenced associations between the development of SRM and functional *CYP2D6* SNP. Frudakis *et al.* evaluated the frequency of the nonfunctional *CYP2D6*4* allele (defined by the rs3892097, c.506-1G>A) in a case-control study ($n = 263$ samples, $n = 388$ SNPs) where 263 patients were followed for the possible development of “muscle event”. In their study, they highlighted that the frequency of the *CYP2D6*4* allele was about 50% in patients developing SRM but only 28% in controls. Even though poorly implicated in ATV metabolism, the authors suggested a possible implication of CYP2D6 in the metabolism of ATV [298].

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Paraoxonases play a key role in detoxifying the body from an ATV lactone accumulation (Figure 1.17). Following this idea, Riedmaier *et al.* studied the association of polymorphisms in *PON1* (–108T>C, –832G>A, and –1741G>A) and in *PON3* (–4984A>G, –4105G>A, –1091A>G, –746C>T, and F21F) with an eventual protein function modulation. They showed that *PON1* expression modulation is mainly ruled by the three polymorphisms linked to the gene promoter which significantly influence the ATV lactone hydrolysis. No polymorphism was identified to alter *PON3* activity where its expression appears to be regulated by non-genetic factors [259].

ii. Phase II metabolism enzymes

The reversed reaction of the lactone detoxification is mainly controlled by the UDP glucuronosyltransferases, *UGT1A3* and little by *UGT1A1*, metabolizing ATV-acid in its lactone form. Amongst others, the polymorphisms *UGT1A1**28 (rs3064744, c.868-6787-868-6786ins(Tan)) and *UGT1A3**2 (rs4663969, c.867+16674C>A) were highlighted by Riedmaier *et al.* They showed that both *UGT1A1**28 and *UGT1A3**2 were associated with an increased activity of ATV lactonization catalyzation *in vitro* and *in vivo* [258]. Observations on *UGT1A3**2 were supported by Cho *et al.* on a cohort of 23 healthy volunteers receiving 20mg of ATV daily. They indeed showed significant increase in ATV lactonization and a lower implication of *UGT1A1**28 mainly because of a lower expression levels of the enzyme compared to *UGT1A3* [299] Contrarily, Stormo *et al.* found that both variants *UGT1A1**28 and *UGT1A3**2 were associated with 41% and 18% reduction in ATV lactone plasma concentration compared to the WT protein, respectively [300].

Summary table of the effects of the highlighted SNPs on ATV PK, efficacy and SRM development.

		SNPs	Frequency	Consequences	
				Clinical	Observed
Efflux	ABCB1	rs2032582 (c.2677G>T/A, p.Ala893Ser/ Thr)	AFR: 0.02-0 EAS: 0.37-0.06 SAS: 0.59-0.05 EUR: 0.41- 0.02	GC haplotype : ATV less efficient to ↓ LDL-C in women [192] Women c.3435C homozygous : lower LDL-C ↓ [192] Consequences of lower ATV circulating concentrations. In 3435T : greater ↓ in LDL-C [268] and ↑ of C _{max} [269]	PK modulation and ATV efficacy
		rs1045642 (c.3435C>T, p.Ile1145=)	AFR: 0.799 EAS: 0.632 SAS: 0.395 EUR: 0.466		
		rs2373588 (g.194405C>T)	AFR: 0.170 EAS: 0.387 SAS: 0.000 EUR: 0.041	Variant more frequent in cases experiencing SRM [270]	SRM
	ABCC1	rs45511401 (c.2012G>T, p.Gly671Val)	AFR: 0.011 EAS: 0.000 SAS: 0.016 EUR: 0.056	No association with LDL-C reduction [209], no effect on ATV clearance [51]. <i>In vitro</i> , counteraction of SLCO2B1 influx and increase ABCC1 activity with SNP [156]	PK modulation and ATV efficacy
	ABCC2	rs717620 (c.-24C>T in 3' UTR)	AFR: 0.058 EAS: 0.212 SAS: 0.113 EUR: 0.200	H12 haplotype (– 24T/1249G/3972T) is at ↑ risk than H2 haplotype (–24C/1249A/3972C) to switch statin therapy [274]	SRM
		rs2273697 (c.1249G>A, p.Val417Ile)	AFR: 0.183 EAS: 0.088 SAS: 0.278 EUR: 0.197		
		rs3740066 (c.3972C>T, p.Ile1324=)	AFR: 0.259 EAS: 0.230 SAS: 0.325 EUR: 0.370		
	ABCC4	rs3742106 (c.*38T>G in 3' UTR)	AFR: 0.322 EAS: 0.511 SAS: 0.494 EUR: 0.388	↓ plasma concentration of ATV and ATV metabolites due to ↓ efflux activity [275]	PK modulation
	ABCG2	rs2231142 (c.421C>A, p.Gln141Lys)	AFR: 0.027 EAS: 0.307 SAS: 0.093 EUR: 0.104	In homologous: ↑ AUC _{0-∞} and C _{max} [129, 247]	No significant effect on PK [277] No change in LDL-C response [278]

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				In homo- and heterozygous, 2.9 more risks of developing SRM [279]		SRM
Influx	SLCO1B1	rs4149056 (c.521T>C, p.Val174Ala)	AFR: 0.030 EAS: 0.125 SAS: 0.050 EUR: 0.159	In variant carriers: ↑ ATV, 2-OH ATV concentrations [128], ↑ AUC and C _{max} [129, 130]	No association of variant carriers and LDL-C response [121]	PK modulation and ATV efficacy
				↑ of the risk to develop SRM [51, 128, 131, 132]		SRM
		rs2306283 (c.388A>G, p.Asn130Asp)	AFR: 0.774 EAS: 0.752 SAS: 0.476 EUR: 0.403	No significant impact on ATV PK [121]		PK modulation
		rs11045818 (c.411G>A, p.Ser137=)	AFR: 0.036 EAS: 0.000 SAS: 0.043 EUR: 0.161	No significant impact on ATV PK [121]		PK modulation
	SLCO1B3	rs57585902 (c.439A>G, p.Thr147Ala)	AFR: 0.031 EAS: 0.000 SAS: 0.000 EUR: 0.000	No impact of the haplotype on ATV transport [148]		PK modulation
		rs60140950 (c.767G>C, p.Gly228Ala)	AFR: 0.026 EAS: 0.001 SAS: 0.070 EUR: 0.157			
		rs2043949386 (c.1559A>C, p.His520Pro)	na			
		rs4149117 (c.334T>G, p.Ser112Ala)	AFR: 0.426 EAS: 0.725 SAS: 0.905 EUR: 0.851	No effect on ATV clearance, SRM development or LDL-C reduction [51]		PK modulation, ATV efficacy and SRM
	SLCO2B1	rs12422149 (c.935G>A, p.Arg312Gln)	AFR: 0.091 EAS: 0.326 SAS: 0.218 EUR: 0.104	In 935A : slight [155] to significant [156] ↓ of <i>SLCO2B1</i> ATV uptake. Tendency of ↓ ATV concentrations in muscle [154]	No effect on ATV clearance [51]	PK modulation
Phase I	CYP3A4	rs35599367 (*22) (c.522-191C>T in intron 6)	AFR: 0.009 EAS: 0.000 SAS: 0.000 EUR: 0.044	↓ metabolism activity leading to ↓ ATV doses [284-287]		PK modulation

Phase I	PPARA	rs4253728 (c.209-1003G>A in intron 4)	AFR: 0.062 EAS: 0.001 SAS: 0.000 EUR: 0.254	↑ in unmetabolized circulating ATV [286]	PK modulation
		mi-21, miR-27b, miR-206	na	Associated with SRM development [288-292]	SRM
	POR	rs1057868 (*28) (c.1508C>T, p.Ala503Val-)	AFR: 0.183 EAS: 0.374 SAS: 0.354 EUR: 0.288	↑ CYP3A activity [293] with a tendency ↓ LDL-C and cholesterol [294]. In homozygous, ↑ cholesterol levels [295]	ATV efficacy
	CYP3A5	rs776746 (*3) (c.219-237A>G in intron 3)	AFR: 0.702 EAS: 0.286 SAS: 0.000 EUR: 0.071	Homozygous at risk of SRM, reduction in CYP3A5 activity [296, 297]	PK modulation and SRM
	CYP2D6	rs3892097 (*4) (c.506-1G>A)	AFR: 0.080 EAS: 0.003 SAS: 0.104 EUR: 0.196	Variant carriers are at ↑ risk of SRM [298]	SRM
	Phase II	rs3064744 (*28) (c.868-6787_868-6786ins(Tan))	AFR: 0.45 EAS: 0.13 SAS: 0.41 EUR: 0.32	↑ of ATV lactonization, ↑ risks of SRM [258] ↓ of ATV lactonization [300]	PK modulation and SRM
		rs4663969 (*2) (c.867+16674 C>A)	AFR: 0.541 EAS: 0.338 SAS: 0.000 EUR: 0.397	↑ of ATV lactonization, ↑ risks of SRM [258, 299] ↓ ATV lactonization [300]	PK modulation and SRM

Table 1.14. Summary of the SNPs with their reported effects on ATV influx and efflux transporters as well as on the phase-I and -II metabolism enzymes. The frequency data are issued from gnomAD v2.1.1 and Ensembl [139-142]. AFR: African, EAS: East Asian, SAS: South Asian, EUR: European and AMR: American.

B. Basal transporters expression levels

The ATV PK and the development of a related muscular toxicity could be explained by the natural inter-individual variability in the transporters' RNA and protein expression. It has indeed been demonstrated in the Chapter IV of this manuscript that there is a high variability in mRNA expression levels that was observed between the volunteers, for a same gene. Those results were globally confirming the GTEx opensource data available on the

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Human Protein Atlas website [301]. Individuals expressing more importantly some transporters might impact the ATV PK and have an effect on ATV related side effects.

The source of inter-individual variability in the transporters' expression was investigated by Nies *et al.* Directly linked to the pharmacogenetics, they highlighted that influx transporters' RNA and protein expression levels were dependent of some genetic factors. They indeed shown that the variant rs2306283 (c.388A>G) in *SLCO1B1* was associated with a significant increase of its protein expression in the hepatocytes. Contrarily, the variant rs4149056 (c.521T>C) affecting the same gene and associated with SRM development, did not modify the protein levels [100, 128]. They interestingly associated the variant c.388A>G with a decreased ATV AUC and the variant c.521T>C with an increased ATV AUC, suggesting that, in addition to the non-synonymous mutation, protein expression might also play a role in the drug disposition. Contrarily, they showed that *SLCO2B1* ARN hepatic expression profile is mostly ruled by transcription factors, while genetic factors only account for a small proportion of the variability. Genetic, non-genetic and transcription factors explained 59% and 55% of *SLCO1B1* and *SLCO1B3* expression variability, respectively. However, only 24% of those parameters explained *SLCO2B1* variability. Other factors like cytokines or microRNAs might be considered as other causes of the variability [100]. In the same study guided by Nies *et al.*, they also demonstrated that the expression of *SLCO1B1*, *SLCO1B3* and *SLCO2B1* RNA and protein levels was partly regulated by non-genetic factors. Among them, alcohol consumption was the most significantly associated with reduced *SLCO1B1* and *SLCO2B1* hepatic RNA expression levels [100]. Depending on the dose, the effects of alcohol on SRM development are then either a combination of a reduced influx transporter expression and an impairment in hepatic functions or an evolution from the first stage to the second. Other research groups such as Medwid *et al.* observed a significant decrease of the cell surface protein expression of *SLCO2B1* variants rs142693902, rs35199625, rs12422149 and rs2306168 by 51, 72, 37 and 83% compared to WT *SLCO2B1*, respectively [153]. Meyer zu Schwabedissen *et al.* also investigated that hypothesis and found that the variant rs2273697 (c.1249G>A, p.Val417Ile) in *ABCC2* significantly reduces the RNA expression in preterm placental tissue. It was associated with a tendency of a reduced protein expression in this tissue [220]. Along the same line, it was demonstrated that *ABCG2*

variant rs7699188 (-15994C>T) was associated with a significantly increased RNA expression in multiple tissues whereas variant -15622C>T was associated with a reduced RNA expression in the intestines [302].

In another register, a study highlighted that the DNA methylation of *SLCO1B3* promoter was linked to its expression. Indeed, in cancerous cell lines where *SLCO1B3* is highly methylated and not expressed (Caco-2 and HepG), incubating them with 5-aza-2'-deoxycytidine induced the expression of the transporter by 14-fold in Caco-2 and by 18-fold in HepG cells [303].

C. Drug-drug interactions

Numerous drug-drug interactions (DDI) have been described to modulate ATV PK, which in some cases influence the SRM development. Alike genetic polymorphisms, inducers and/or inhibitors of influx and efflux transporters as well as metabolization enzymes influence the response to a treatment, modulating its PK. Inhibitors of the influx and efflux transporters have been listed in Table 1.4 and in Table 1.8 of this first chapter, respectively. Of note, referenced protein inducers are less numerous and poorly studied.

ATV is being mainly metabolized by *CYP3A* and more precisely by *CYP3A4*, a concomitant treatment with inducers or inhibitors of the metabolization enzyme might thus result in an elevation or reduction of the ATV circulating levels, modifying the efficacy and the safety of the drug [304]. The strong inducers include carbamazepine, phenobarbital or rifampin (rifampicin) and the moderate inducers include, among others, efavirenz, nafcillin or lorlatinib [305]. Lots of strong *CYP3A4* inhibitors are used in the treatment of HIV (e.g. atazanavir, darunavir or indinavir) or used to treat antifungal infections (e.g. itraconazole or voriconazole) or bacterial infections (e.g. clarithromycin). Moderate inhibitors include, among others, erythromycin, verapamil and imatinib [304, 305]. It has even been demonstrated that there is an elevated risk of SRM development when ATV is taken concurrently with azole antifungals or HIV protease inhibitors, increasing ATV C_{max} and $AUC_{0-\infty}$ by inhibition of *CYP3A4* [306, 307].

To illustrate the DDI affecting the phase-II metabolization enzyme, the fibrates, and more particularly the gemfibrozil, taken concomitantly to a treatment with ATV is the best candidate. In the past, patients with unachieved LDL-C and/or total cholesterol levels in

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mixed hyperlipidemia have been advised to take gemfibrozil in supplementation to their ATV treatment [81]. Gemfibrozil however led to an increase of ATV AUC, increasing the risk of serious myotoxicities that might evolve in rhabdomyolysis [308]. The underlying toxicity mechanism comes from the competition of both gemfibrozil and ATV for *UGT1A1* and *UGT1A3*, limiting the glucuronidation of ATV which will increase the circulating ATV levels [260]. It has also been described that gemfibrozil inhibits the hepatic uptake of statins by inhibition of OATP1B1 and the metabolism of statins, particularly cerivastatin, through CYP2C8. These are additional mechanisms lead to increased statin plasma concentrations when taken concomitantly with gemfibrozil [309].

Finally, inhibitors of OATPs might also modulate the PK of statins. The literature has indeed evidenced that all statins are the substrates of OATP1B1 transporter, and the co-prescription of inhibitors of OATP1B1, like cyclosporine, elevates their plasma concentrations and muscle toxicity [306]. In a recent popPK study, L-thyrosine, an OATP2B1 inhibitor has been associated with decreased ATV apparent clearance of ATV, reflecting a potential decrease hepatic uptake of the drug with the concomitant use of this inhibitor [51].

CHAPTER II - CONTEXTUALIZED OBJECTIVES

CHAPTER II – CONTEXTUALIZED OBJECTIVES

What is the problematic?

Cardiovascular diseases (CVD) are a major public health issue worldwide, that cause the death of more than 4 million European citizens each year. They can be attributable to many factors, however, high blood levels of low density (LDL-C) and very low density (VLDL-C) lipoproteins cholesterol that promote atherosclerotic plaques have a substantial impact in the onset of CVD [2, 3, 24]. Atorvastatin (ATV), belonging to the statins drug class, has proven its high efficacy to reduce LDL-C by up to 50% at high doses [30]. ATV has been demonstrated as an effective strategy to reduce the risk of CVD in fatal or nonfatal strokes, both in primary and secondary prevention [24]. The undeniable effectiveness of statins has established them as the first-line therapy in Europe, with ATV being the most prescribed drug in its category in Belgium [3, 35].

While the drug is generally well-tolerated, 7 to 15% of the patients suffer from ATV-induced muscle side-effects [51]. These side effects can range from mild muscle discomfort to cramps, and in very rare cases, to rhabdomyolysis [57, 255]. Despite the numerous studies on the issue, the mechanisms leading to ATV-related muscle toxicity are not yet fully understood. Moreover, predicting their occurrence is challenging, as it varies significantly among individuals. Altogether, the side effects directly affect patients' adherence to the treatment and consequently reduce the effectiveness of ATV in preventing the development of CVD [25].

What is the state of the art?

It is known that the toxicity is dose- and concentration-dependent, with patients under high ATV doses being at higher risk to develop muscle side-effects [58]. In patient suffering from muscle side-effects, it has been demonstrated that the ratio of ATV lactone to ATV acid is correlated with the risk of developing this discomfort. Moreover, it was shown that plasma and muscle concentrations correlate better for lactone metabolites when compared to the corresponding acid forms. Local pharmacokinetics (PK) is thus assumed to play a key role in the onset of ATV-induced muscle side effects. Given the lipophilic nature of ATV, the drug can cross cell membranes through passive diffusion but to a limited extent compared to the

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lactone forms. Furthermore, it is widely referenced that ATV PK is ruled by influx and efflux transporters to penetrate the body compartments [81, 129, 174, 247, 275]. According to opensource databases, those transporters are expressed in various tissues including the skeletal muscle tissue. It is reported that among influx transporters, *SLCO2B1* is the only one expressed in this cell type. Regarding the efflux transporters, *ABCC1* is the most represented but *ABCC4* and *ABCG2* are also found at lower intensity levels [301].

The high inter-individual variability in the development of ATV-related toxicity suggests that the pharmacogenetics also plays a major part in its occurrence. For instance, the rs4149056 variant (c.521T>C, p.Val174Ala) in *SLCO1B1* has been associated with increased ATV circulating blood metabolites that results from a decreased hepatic uptake due to a defect in the influx activity of the transporter [128-130]. Due to the increased plasma exposure, the variant was demonstrated to be associated with an increased risk to develop ATV-related muscle side-effects [100, 128, 131, 132]. In a lesser extent, the variant rs2231142 (c.421C>A, p.Gln141Lys) in *ABCG2* was also linked to an increase in ATV $AUC_{0-\infty}$ and C_{max} , associated with a 2.9-fold increased risk of statin-related muscle side effect development [129, 247]. These two examples illustrate SNPs that have a systemic impact on ATV PK, as *SLCO2B1* and *ABCG2* are primarily expressed in hepatocytes [301]. Although, little is known about the ATV PK in the skeletal muscle tissue, Knauer *et al.* demonstrated that overexpression of influx and efflux transporters, presumed to be expressed in skeletal muscle tissue, in Hela transfected models induced a modulation of the ATV intracellular accumulation. They also showed that at high ATV doses, transfection-induced overexpression of the efflux transporter *ABCC1* in commercialized myotubes, significantly reduced the toxicity induced by the drug [174].

The literature suggests an intricate interplay between ATV-related toxicity, pharmacokinetics governed by influx and efflux transporters, and the influence of pharmacogenetics observed at the systemic level.

What is the scientific hypothesis?

The research conducted in this thesis is centered on understanding the cellular ATV PK. This involves exploring the role of influx and efflux transporters expressed in skeletal muscle tissue on the intracellular accumulation of this molecule. The hypothesis posits that the expression and activity of these transporters, which vary among individuals and might be affected by genetic polymorphisms, could modulate the intracellular accumulation of ATV in muscle cells and, consequently, influence the onset of side effects. The focus is set on a better understanding the local ATV PK rather than its systemic PK. Therefore, the present study aims to contribute by addressing the following question:

***“What is the implication of the efflux and influx transporters
in ATV cellular transport?”***

What is the research strategy?

The research strategy is divided into two complementary parts. The first one (Figure 2.1, Research article I in Chapter III) comprises fundamental investigations, aiming at assessing the net impact of overexpressing the influx (SLCO2B1) and efflux (ABCC1) transporters, as well as the effect of functional SNPs, on ATV transport. Initially, *in vitro* HEK293 transfected cell models were developed, overexpressing either one of the transporters or both. Subsequently, similar models were developed, where we introduced genetic variants in the coding sequences of *SLCO2B1* (rs12422149 (c.935G>A p.Arg312Gln)) and *ABCC1* (rs45511401 (c.2012G>T, p.Gly671Val)). Protein expression measurement and the visualization of its location in the cells was facilitated by the inclusion of fluorescent tags grafted onto the protein sequence of the plasmids. The uptake and efflux of ATV were characterized in the different models developed with increasing doses of the drug to explore the net impact of wild-type and variant carriers on ATV intracellular transport.

Fundamental investigations

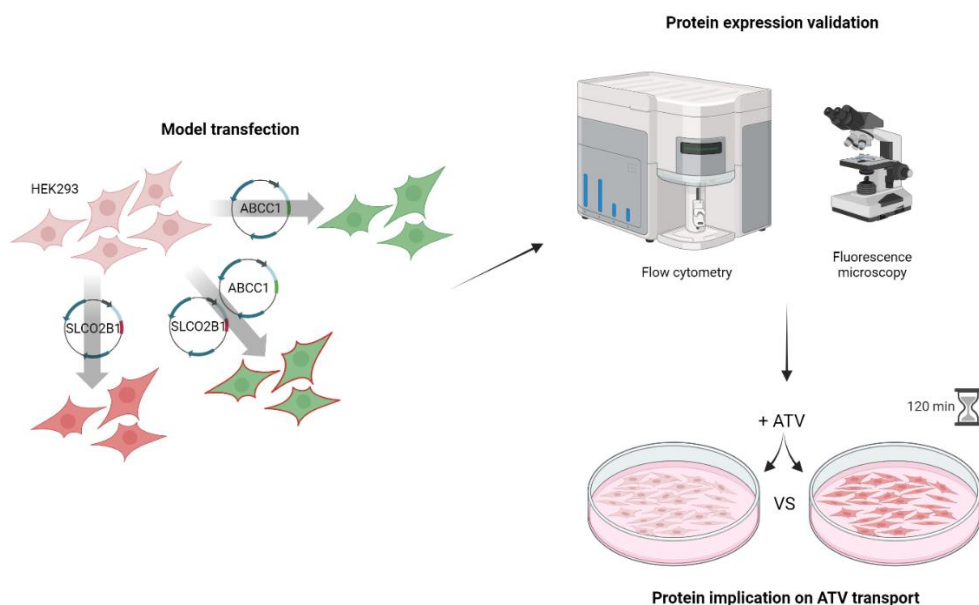


Figure 2.1. Illustration of the fundamental part of the thesis project. With, first, the development of recombinant HEK293 models overexpressing the proteins identifiable through red or green fluorescent tags integrated at the end of their sequence. This characteristic was used in flow cytometry and fluorescence microscopy to confirm the expression and the localization of the proteins in the cell models. Subsequently, the cell lines were utilized to assess the implication of the transporters in the intracellular ATV accumulation.

Although we do not possess information about whether the levels obtained after *in vitro* differentiation accurately represent those found in myotubes *in vivo*, the second part of the project (Figure 2.2, Research article II in Chapter IV) consists in the translational investigations using a model that is believed to better mimics the physiological situation. This phase was conducted on primary cultures of myotubes derived from skeletal muscle biopsies of twelve volunteers. *In vitro*, the biopsy explants were plated to allow the proliferation of the satellite cells further purified on a magnetic sorting system (MACS). After proliferation, the myoblasts were able to fuse and differentiate into myotubes, used

for the experiments. The RNA expression of four efflux (*ABCC1*, *ABCC2*, *ABCC4* and *ABCG2*) and three influx (*SLCO2B1*, *SLCO1B1* and *SLCO1B3*) transporters was measured in the myotubes. The transport of ATV was characterized in this cell type, expressing the transporters at biological levels. Simultaneously, siRNAs were used to silence the expression of the most expressed efflux (*ABCC1* and *ABCG2*) and influx (*SLCO2B1* and *SLCO1B1*) transporters to assess the impact of their repression on ATV intracellular accumulation.

Translational investigations

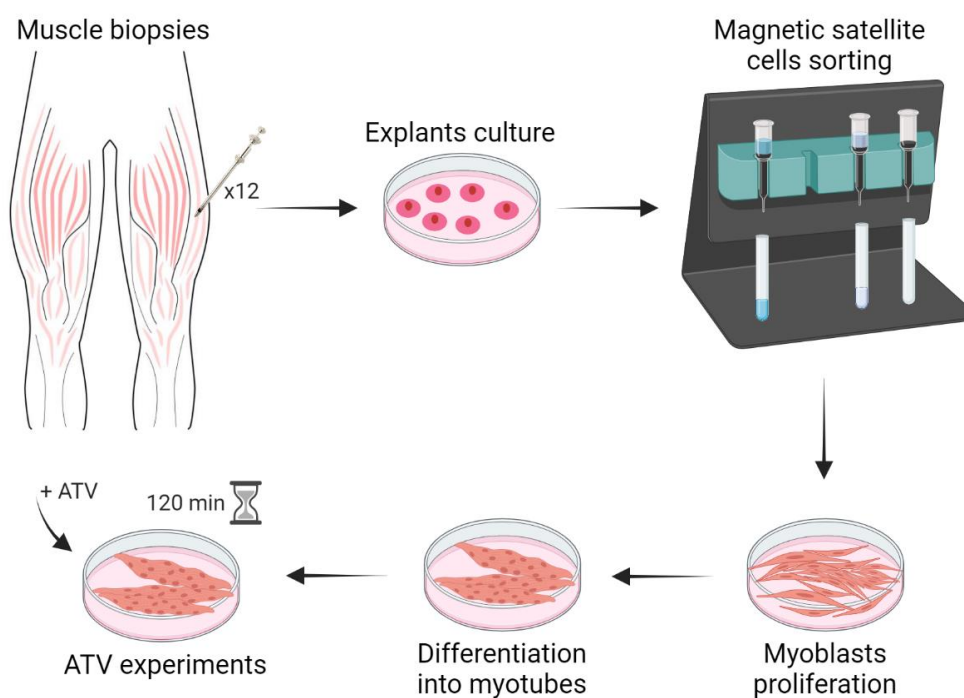


Figure 2.2. Illustration of the translational part of the thesis project. Muscle biopsies from twelve volunteers were excised then plated in Petri dishes to harvest the satellite cells and subsequently sort them with a MACS. For the ATV accumulation experiments, after a proliferation phase, the cells were subsequently differentiated into myotubes.

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CHAPTER III - FUNDAMENTAL INVESTIGATIONS

This chapter is adapted from our research article by Hoste *et al.*, entitled “**Genetic Polymorphisms in SLCO2B1 and ABCC1 Conjointly Modulate Atorvastatin Intracellular Accumulation in HEK293 Recombinant Cell Lines**”, published in *Therapeutic Drug Monitoring* in June 2023.

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Genetic Polymorphisms in *SLCO2B1* and *ABCC1* Conjointly Modulate Atorvastatin Intracellular Accumulation in HEK293 Recombinant Cell Lines

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1. ABSTRACT

Background: Although Atorvastatin (ATV), is generally well tolerated, patients may report muscle complaints. These are difficult to predict owing to high inter-individual variability. Such side effects are linked to intramuscular accumulation of ATV. This study aimed to investigate the relative role of transporters expressed in muscle tissue in promoting or limiting drug access to cells. The impact of common single nucleotide polymorphisms (SNP) in *SLCO2B1* coding for OATP2B1 and *ABCC1* coding for MRP1 on ATV transport was evaluated.

Methods: HEK293 cells were stably transfected with plasmids containing cDNA encoding wild-type or variant *SLCO2B1* and/or *ABCC1* to generate single and double stable transfectant HEK293 recombinant models overexpressing variant or wild-type OATP2B1 (influx) and/or MRP1 (efflux) proteins. Variant plasmids were generated by site-directed mutagenesis. Expression analyses were performed to validate recombinant models. Accumulation and efflux experiments were performed at different concentrations. ATV was quantified by LC-MS/MS, and kinetic parameters were compared between single and double HEK transfectant expressing wild-type and variant proteins.

Results: The results confirm the involvement of OATP2B1 and MRP1 in ATV cellular transport because it was demonstrated that intracellular accumulation of ATV was boosted by OATP2B1 overexpression, whereas ATV accumulation was decreased by MRP1 overexpression. In double transfectants, it was observed that increased ATV intracellular accumulation driven by OATP2B1 influx was partially counteracted by MRP1 efflux. The c.935G>A SNP in *SLCO2B1* was associated with decreased ATV OATP2B1-mediated influx, whereas the c.2012G>T SNP in *ABCC1* seemed to increase MRP1 efflux activity against ATV.

Conclusions: Intracellular ATV accumulation is regulated by OATP2B1 and MRP1 transporters, whose functionality is modulated by natural genetic variants. This is significant because it may play a role in ATV muscle side-effect susceptibility.

Key Words: atorvastatin, pharmacogenetic, influx and efflux transporters, adverse drug reactions

2. INTRODUCTION

Atorvastatin (ATV), an HMG-CoA reductase inhibitor, commercialized under the name of statins, has proven its efficacy in reducing increased blood levels of low-density lipoprotein cholesterol (LDL-C) and limiting the risk of cardiovascular disease [1, 2]. Although generally well tolerated, 5 to 10% of the patients may suffer from muscle complaints, a relatively uncomfortable side effect that can eventually lead to spontaneous poor patient compliance [3-5]. As an HMG-CoA reductase inhibitor, ATV competitively inhibits the mevalonate pathway implicated in cholesterol synthesis [6]. Although ATV-mediated HMG-CoA reductase inhibition is related to its effect on cholesterol synthesis, it is believed to also account for its adverse effects, among which are muscular complaints. In fact, the ATV-mediated inhibition of the mevalonate pathway and the consequent downstream metabolite depletion leads to a joint reduction of their respective physiological activities which could possibly explain the ATV-related muscular side effects [7]. ATV-mediated HMG-CoA reductase inhibition and the resulting metabolite depletion are known to be dose-dependent [1]. This dose-dependent response suggests that understanding ATV intracellular pharmacokinetics (PK) and the factors affecting it is necessary to unravel the reasons for treatment response variabilities and susceptibility to muscle side effects. In particular, the fact that statin-associated muscle symptoms are specific to skeletal muscle tissue, and dose and drug potency dependent [8-10], provides a rationale for exploring the importance of factors affecting intramyocyte concentrations. ATV can enter myocytes where toxicity occurs through passive diffusion. However, some data also suggest that active transport is a preponderant mechanism [11]. The activity of transporters expressed in skeletal muscle tissue is assumed to modulate drug concentrations directly within myocytes through both drug efflux and influx. Among the efflux transporters implicated in ATV PK, MRP1, encoded by *ABCC1* (ATP-binding cassette sub-family C, member 1), is one of the most expressed, whereas OATP2B1, encoded by *SLCO2B1* (solute carrier organic anion sub-family 2B, member 1) is, to the best of our knowledge, the only drug influx transporter involved in ATV transport and expressed in skeletal muscle tissues [12]. This suggests that these transporters play a role in protecting or sensitizing myocytes from statin toxicity.

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However, the correlation between transporter activity and ATV cellular disposition has been poorly explored. Elucidating the potential impact of functional single nucleotide polymorphisms (SNPs) on the role of these transporters in driving the drug across membranes and determining drug cellular accumulation may explain interindividual differences in the risks of muscle side effects. *ABCC1* is located on chromosome 16p13.11, and contains 34 exons (NG_028268.2). This efflux transporter transports various molecules such as ions, glutathione or xenobiotics [13]. *ABCC1* expression is relatively ubiquitous. However, its expression is significant in the lungs, on the basolateral membranes of proximal renal tubular cells, and epithelial cells of the small intestine. *ABCC1* is not expressed in the liver [14]. *ABCC1* is highly polymorphic. Among others, the rs45511401 (c.2012G>T, Gly671Val) SNP is a non-synonymous coding SNP located close to the first nucleotide-binding domain of the protein [15]. OATP2B1 is an influx drug carrier that transports endogenous (e.g., estrone-3-sulfate and pregnenolone sulfate) and exogenous (e.g., ATV and rosuvastatin) substrates. Its coding gene (*SLCO2B1*) is located on chromosome 11q13.4, which contains 16 exons (NG_027921.1) [16]. This influx transporter is mainly expressed on the basolateral hepatic membranes, facilitating the extraction of endogenous substrates and drugs from the blood to the hepatocyte for further metabolism [17, 18]. It is also expressed in the skeletal muscle tissue, blood-brain barrier, kidney, small intestine, and, to a lesser extent, placenta [19]. Because the protein is not very well characterized, little data are available. However, the non-synonymous SNP rs12422149 (c.935G>A, Arg312Gln) in *SLCO2B1* has been associated with increased simvastatin plasma levels and reduced rosuvastatin efficiency, suggesting a significant impact of this SNP on the PK of its substrates [20, 21]. However, to date, no data on ATV are available.

Both polymorphisms are commonly found in the general population (Table 3.1). The minor allele frequency of rs45511401 in *ABCC1* is higher in European population (4.6%) than in the American (2%), South Asian (1.1%) and African (0.5%) populations, with a global minor allelic frequency of approximately 2%. The minor allele frequency of rs12422149 in *SLCO2B1* is higher globally (21%), with a very high frequency in American (36.5%) and Southeast Asian

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populations (26.3% and 32%, respectively) and a lower frequency in African and European populations (9.2 and 9.5%, respectively) [22, 23].

The goal of this study was to assess the activity of both transporters and the functional effects of 2 common nonsynonymous coding SNPs on ATV cellular transport. In this context, *in vitro* recombinant models overexpressing the transporters either separately (single transfectants) or simultaneously (double transfectants) were developed by stably transfecting HEK293 cells.

rs45511401 in <i>ABCC1</i>		rs12422149 in <i>SLCO2B1</i>	
Global	0.015	Global	0.210
European	0.046	European	0.095
American	0.020	American	0.365
African	0.005	African	0.092
South Asian	0.011	South Asian	0.263
East Asian	0	East Asian	0.320

Table 3.1. Minor Allele Frequency among ethnicities for rs45511401 in *ABCC1* and rs12422149 in *SLCO2B1* according to Ensembl.org

3. MATERIALS AND METHODS

Materials

Atorvastatin (ATV) was purchased from Pfizer (New York, NY, USA) and the d5-ATV calcium salt from Alsachim (CAS number: 222412-82-0). Gentamicin (G418), hygromycin B, dimethyl sulfoxide (DMSO), ampicillin and kanamycin were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

Control and transfected Human Embryonic Kidney cells (HEK 293, ATCC N°CRL-1573) were grown in Dulbecco's Modified Eagle Medium with high glucose, L-glutamine and sodium pyruvate (DMEM GIBCO®) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (ThermoFisher Scientific Waltham, MA). The cells were cultured at 37°C and 5% CO₂. For re-culturing, the cells were detached with enzyme-free cell dissociation buffer (ThermoFisher Scientific).

Generation of *SLCO2B1*_{935G} and *SLCO2B1*_{935A} plasmids

The expression vector pCMV-V-OFPSpark®, which encodes for the wild-type OATP2B1 protein (*SLCO2B1*_{935G}) and a C-terminal fluorescent red tag (OFPSpark®) was purchased from Bio-Connect Life Sciences (cat. HG18756-ACR; Huissen, the Netherlands). The construct contained sequences coding for kanamycin and hygromycin B resistance genes, allowing further bacterial and eukaryotic cell selection, respectively. The variant plasmid expressing *SLCO2B1*_{935A} (rs12422149) was generated by site-directed mutagenesis by polymerase chain reaction (Icycler IQ; BioRad, Hercules, CA) using the Phusion Site-Directed Mutagenesis kit (Waltham, MA) following the manufacturer's protocol. The back-to-tail primers phosphorylated in 5'-OH, including the point mutation, were 5'-CAG TTT CGG CAA AAG GTC TTA G-3' (forward) and 5'-AAG CTC ACG TTT TTC CTT G-3' (reverse) and were purchased from Eurofins Scientific société européenne (Ebersberg, Germany). Plasmid transformation and amplification were performed in dh5α *Escherichia coli* strains using heat shock. The product was purified using a PureLink™ HiPure Plasmid Filter Maxiprep (ThermoFisher Scientific, Waltham, MA). The amplicon was sequenced using the Sanger method (Eurofins,

Germany) to confirm the introduction of the point mutation c.935G>A into the coding nucleotide sequence of *SLCO2B1* (see Supplemental Data, Figure S3.1a, b, Supplemental Digital Content 1, <http://links.lww.com/TDM/A619>).

Generation of *ABCC1*_{2012G} and *ABCC1*_{2012T} plasmids

The expression vector pcDNA3.1-eGFP was kindly provided by Dr. Susan Cole [15] codes for the wild-type MRP1 protein (*ABCC1*_{2012G}) with a green fluorescent tag (GFP). The construct contained sequences coding for ampicillin and gentamicin resistance genes, allowing further bacterial and eukaryotic cell selection, respectively. As for *SLCO2B1*, the variant plasmid expressing *ABCC1*_{2012T} (rs45511401) was generated by site-directed mutagenesis by PCR using the Phusion Site-Directed Mutagenesis kit. The back-to-tail primers phosphorylated at the 5'-OH, including the point mutation, were 5'-ATC CCC GAA G_T GCT TTG GTG-3' (forward) and 5'-GGA GAA GGT GAT GCC ATT C-3' (reverse). The product was amplified and purified under the same conditions as previously, and the variant was successfully introduced into the coding sequence of *ABCC1* (see Supplemental Data, Figure S3.1c, d, Supplemental Digital Content 1, <http://links.lww.com/TDM/A619>).

Generation of stable recombinant cell lines

For single transfectants, overexpressing either WT or the variant (var) OATP2B1 protein, i.e. HEK_{OATP2B1WT} and HEK_{OATP2B1var}, 5×10^5 HEK293 cells/well were seeded in 12-well plates the day before transfection with the Lipofectamine 3000 Transfection Kit (Invitrogen, Carlsbad, CA) with 1.25µg of plasmid DNA (either *SLCO2B1*_{935G} or *SLCO2B1*_{935A} plasmids). The same method was used to obtain HEK_{MRP1WT} and HEK_{MRP1var} single transfectants. For the double transfectants, 1×10^6 cells overexpressing one of the two proteins (HEK_{OATP2B1WT} or HEK_{OATP2B1var}) were cultured in 6-well plates one day before lipofection. Transfection was then achieved using 2.5µg of plasmid DNA per well (either *ABCC1*_{2012G} or *ABCC1*_{2012T} plasmids). The antibiotic selection was performed 72h post-lipofection with hygromycin B and G418 at a final concentration of 0.5 mg/ml and 1 mg/ml to select cells that stably expressed OATP2B1 and/or MRP1 proteins, respectively. Resistant strains were reseeded after 14 days for future development and sorted using flow cytometry.

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Double transfectant models were renamed thereafter, with the model overexpressing both WT transporters, HEK_{OATP2B1WT-MRP1WT}; the model overexpressing *SLCO2B1* WT and *ABCC1* variant, HEK_{OATP2B1WT-MRP1var}; and the model overexpressing *SLCO2B1* variant and *ABCC1* WT, HEK_{OATP2B1var-MRP1WT}. Based on the relatively low frequency of the polymorphisms in the population, the impact of both variants in the double transfectant model HEK_{OATP2B1var-MRP1var} was not investigated (the theoretical frequency of the combination is 0.66%).

Characterization of OATP2B1 and MRP1 protein expression

Flow cytometry

The cells were detached, counted, and centrifuged at 400 g for 5 minutes at 4°C on the day of the experiment. The supernatant was discarded, and the cell pellet was resuspended in 2 mL of sterile FACS-Buffer (Dulbecco's PBS, 1% (vol/vol) decompemented fetal bovine serum, 0.001mol/L EDTA [ThermoFischer Scientific]). Cells were washed twice and resuspended in FACS buffer for analysis with a BD-FACS Verse or sorting with BD FACSaria® III (BD Biosciences, NJ). OFPSpark® (red OATP2B1) and eGFP (green MRP1) fluorescence were excited with a blue laser (488nm) with bandpass filters BP 586/42 and 527/32, respectively. Raw data were analyzed using FlowJo version 10.8.1 (Ashland, OR). Because fluorescent OFPSpark® and eGFP emission spectra partially overlapped, the data were compensated using FlowJo.

All the generated recombinant models were sorted using fluorescence parameters gated at the same level of intensity to ensure similar protein expression. Sorted cells were reseeded in 12-well plates with adequate selective antibiotic concentrations to allow for growth expansion.

Inverted fluorescence microscope

Cells were seeded in Ibidi 4 chambers pH+ μ -slide (Gräefelfing, Germany) 48h before microscopic analysis using Zeiss Observer Z1 (Zeiss, Germany). The fluorescence signals were obtained using a Zeiss Colibri 7 LED light source. Red fluorescence (OFPSpark®) emitted by the tag attached to OATP2B1 was observed with a yellow LED at an excitation wavelength of 555 nm and an emission quadrature bandpass 582/30. The green fluorescence (eGFP) emitted by the tag attached to MRP1 was observed with a blue LED at

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an excitation wavelength of 475 nm and an emission quadrature bandpass 514/30. Images were captured with a Zeiss Axiocam 506 mono and processed using the Zeiss Zen 2 lite.

Characterization of OATP2B1 and MRP1 activity

Atorvastatin intracellular accumulation and efflux kinetics

The day before accumulation assay, the cells (3×10^5) were seeded in 24-well plates coated with poly-L-lysine (Sigma-Aldrich), reaching a confluence of ~90% the next day. On the day of the experiment, 50 μ L of each well was removed and 50 μ L of freshly diluted ATV solution was added. For drug accumulation, cells were incubated for 120 minutes with increasing ATV concentrations (0, 50, 75, 100, 150 and 500nM) whereas a concentration of 75nM was selected for efflux kinetics. All experiments were performed at least twice (N=2) in triplicates (n=3). After ATV incubation at 37°C with 5% CO₂ saturation, the plates were centrifuged (Eppendorf 417R) at 400 g for 5 minutes at 4°C and the supernatant was discarded. Cells were washed three times with 500 μ L of ice-cold D-PBS and either recovered for accumulation experiments. For the efflux kinetics, after a quick centrifugation at 4°C, the supernatant was kept for the quantification then a new medium was added to allow the cells to efflux in drug-free medium for 30, 60, 120, or 300 minutes. The cells were finally resuspended in 500 μ L of D-PBS supplemented with 0.001mol/L EDTA, transferred to a 1.5ml Eppendorf® (Hamburg, Germany), and centrifuged at 400g for 5 minutes at 4°C. The supernatant was discarded, and the cell pellet was stored at -20°C for further extraction. For efflux kinetics, aliquots of media (500 μ l) were collected at each time point to quantify the fraction of drug effluxed and the cell pellet collected using the same method previously described. The efflux ratio was calculated by normalizing the amount of effluxed ATV in the medium at the different time points (30, 60, 120 or 300 minutes) reported on the intracellular amount accumulated at the start of the efflux period (i.e. as quantified in cells after 2hours of accumulation).

Atorvastatin extraction and quantification

Thawed cell pellets were resuspended in 100 μ L of an extraction solvent consisting of a mixture of methanol-water (60:40, v/v) and d5-ATV at a final concentration of 5nM. The samples were vortexed, sonicated for 10 minutes, and centrifuged for 5 minutes at 10,600

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g at room temperature (20°C). The cell pellets were stored at -20°C for protein assay, and supernatants were transferred to an injection vial and dried under nitrogen flow. The residue was resuspended in 30µL methanol before injection into an HPLC-MS/MS system. For ATV media quantification, 500µL of media was added to 1.2mL of ice-cold acetone supplemented with d5-ATV at a final concentration of 5nM. The mixture was vigorously vortexed, stored at -20°C for 2 hours, and then centrifuged at 10,600 g for 10 minutes. The supernatant was then evaporated and resuspended in methanol. Each extract (5µL) was injected into an HPLC-MS/MS system (Xevo TQ-S, Waters, Milford, MA). Analyte separation was performed using a gradient between mobile phase A (MeOH-H₂O (75:25) with 0.1% (v/v) acetic acid) and mobile phase B (MeOH with 0.1% (v/v) acetic acid), and a ultra-performance liquid chromatography column (Ascentis Express C18 column [2.7µm, 150 × 4mm]) equipped with a precolumn. For MS/MS detection, ionized compounds were generated using an electrospray source operated in the positive mode. Detection was performed in the Multiple Reaction Monitoring (MRM) mode using the quantification transitions 564.3 → 440.3 for d5-ATV and 559.2 → 440.2 for ATV. Calibration curves were established between 0.5 and 1000 fmol ATV on the column. In addition, in each run of ATV quantification, we screened our samples for ortho- and para-hydroxy-ATV (575.2>440.2); and for ATV lactone (541.2>448.2) [4]. In all cases, metabolite levels were below the respective detection limits (400 and 500amol for p- and o-OH-ATV respectively and 375amol for ATV lactone), suggesting that the spontaneous conversion of the acid form is limited in our *in vitro* system and cannot explain the observed differences. Data analysis was performed using MassLynx software (Waters). ATV concentrations were normalized to the total protein content.

Protein normalization and statistical analysis

The protein assay was performed in triplicate for each sample with a thawed cell pellet using the DC™ Protein Assay (BioRad, Hercules, CA).

Graphs and statistical analyses were performed using GraphPad Prism 8.4.2. To assess the impact of transporters on the intracellular accumulation of ATV, 2 way-analysis of variance was used to evaluate the impact of two independent factors, that are, ATV incubation

concentrations (accumulation experiment) or efflux time (efflux kinetics), and the cell lines. Bonferroni post-hoc tests were performed for multiple comparisons to determine which means differed from the others. In all cases, the results were considered significant when the *P* value was < 0.05.

4. RESULTS

Generation of stable *SLCO2B1*_{935G} and *SLCO2B1*_{935A} overexpressing cell lines and characterization of OATP2B1 expression level

After transfection of HEK293 cells with pCMV-V-OFPSpark® containing the coding sequence *SLCO2B1*_{935G} or *SLCO2B1*_{935A}, similar OATP2B1 expression was ensured by sorting the cells using a fluorescence activated cell system (FACS) gated on the same level of fluorescence intensity.

The expression levels of OATP2B1 were examined by fluorescence microscopy. The results confirmed that the recombinant cell lines HEK_{OATP2B1WT} and HEK_{OATP2B1var} overexpressed OATP2B1 influx protein. Indeed, bright red fluorescence of the overexpressing cells was observed in HEK_{OATP2B1WT} and variant, whereas no fluorescent signal was observed for the control cells (HEK_{CTRL}) (Fig. 3.1A). Furthermore, a strong encircling fluorescent signal was observed indicating the dominant location of the transporters in the membrane.

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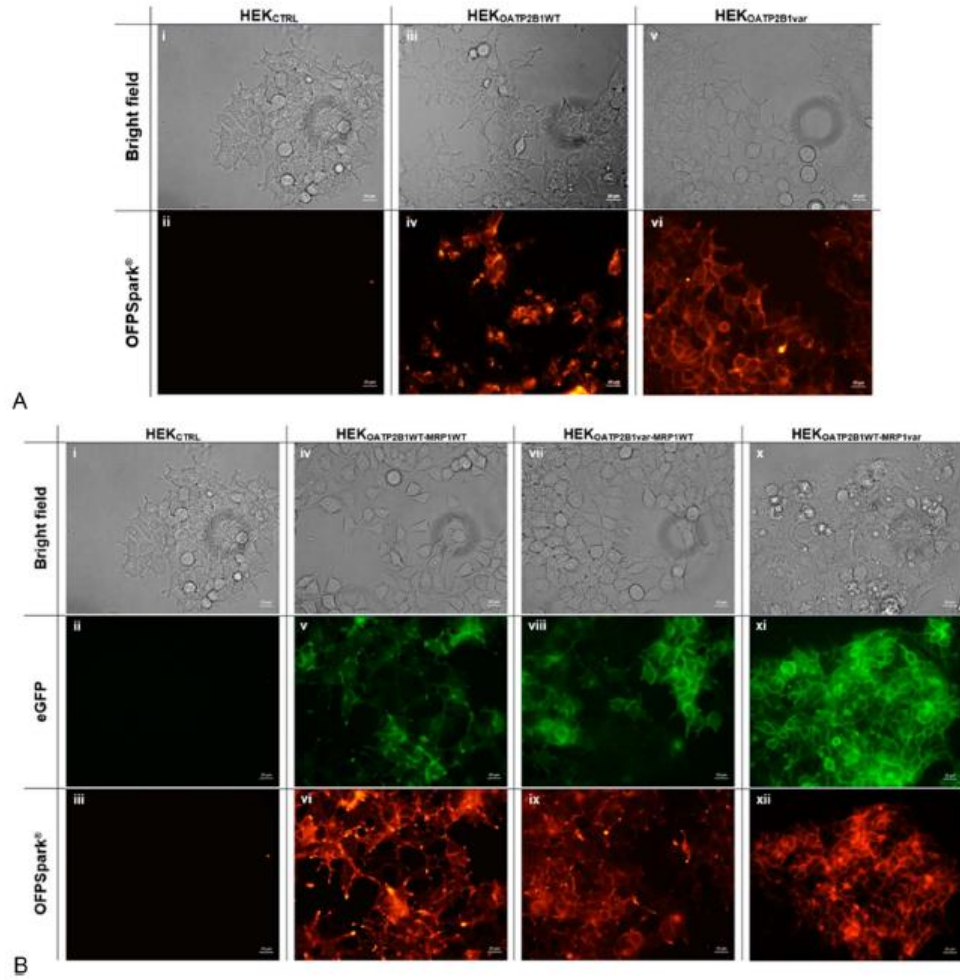


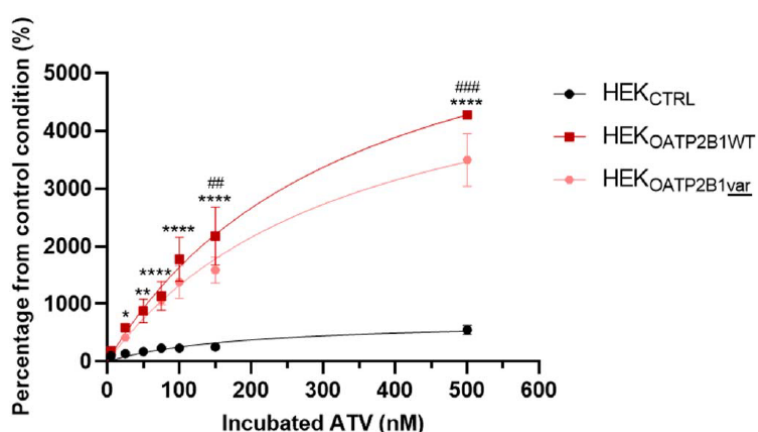
Figure 3.1. Fluorescence microscopy in (A) single transfectant models (i, ii) HEK_{CTRL}, (iii, iv) recombinant HEK_{OATP2B1WT}, and (v, vi) recombinant HEK_{OATP2B1var} in bright field above and in red OFPSpark detection below and fluorescence microscopy in (B) double transfectant models (i–iii) HEK_{CTRL}, (iv–vi) recombinant HEK_{OATP2B1WT-MRP1WT}, (vii–ix) recombinant HEK_{OATP2B1var-MRP1WT}, and (x–xii) recombinant HEK_{OATP2B1var-MRP1var} in bright field above, in green eGFP detection centered, and in red OFPSpark detection below.

Generation of stable *SLCO2B1*_{935G/A} and *ABCC1*_{2012G/T} double transfectant cell lines and characterization of protein expression level

As described in the Material and Methods section, double transfectants were generated by transfecting cells stably expressing OATP2B1 with pcDNA3.1-eGFP *ABCC1* expression vectors (either WT or variant). As for single transfectants, similar protein expression was ensured by sorting the cells by FACS gated at the same level of fluorescence intensity. This was confirmed by inverted fluorescence microscopy, where all double transfectants expressed green and red fluorescence when compared to HEK_{CTRL} cells (Fig. 3.1B). This observation confirmed that both influx and efflux proteins were overexpressed in the double transfectant cell models.

Effect of *SLCO2B1*_{935G} versus *SLCO2B1*_{935A} overexpression on ATV intracellular accumulation

The results presented in Figure 3.2 compare the intracellular accumulation of ATV in HEK_{OATP2B1}WT, HEK_{OATP2B1}var and HEK_{CTRL} cells. After 2 hours of drug incubation, ATV cellular content was significantly increased in HEK_{OATP2B1} WT cells compared to HEK_{CTRL} cells from 25nM onwards (**P* < 0.05 at 25nM, ***P* < 0.01 at 50nM, *****P* < 0.0001 from 75nM of ATV). Interestingly, HEK_{OATP2B1}var showed reduced ATV accumulation compared to its WT counterpart, and the difference was significant at 150nM and 500nM. (##*P* < 0.01 at 150nM and ###*P* < 0.001 at 500nM).



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Figure 3.2. ATV accumulation experiment represented in percentage from the control condition (ATV accumulation in HEK_{CTRL} at the lowest concentration) in single transfectant models HEK_{OATP2B1WT} and HEK_{OATP2B1var} compared with the control cells HEK_{CTRL} (**P* < 0.05, ***P* < 0.01, *****P* < 0.0001 HEK_{OATP2B1WT} compared with HEK_{CTRL} and ##*P* < 0.01, ###*P* < 0.001 HEK_{OATP2B1var} compared with HEK_{CTRL}).

Effect of *SLCO2B1*_{935G/A} and *ABCC1*_{2012G/T} co-expression on ATV intracellular accumulation

After 2 hours of incubation with increasing concentrations of ATV, the percentage of accumulation in HEK293 control cells was lower than that in all other cell lines (Figs. 3.3A, B). By contrast, intracellular accumulation in the single transfectant HEK_{OATP2B1WT} cells was higher than the values observed for all other cell lines, regardless of the conditions.

Interestingly, MRP1WT activity reduced the increased accumulation generated by the OATP2B1WT influx. Indeed, intracellular ATV accumulation in HEK_{OATPWT-MRP1WT} double transfectant was significantly lower than that in HEK_{OATP2B1WT} but still significantly higher than that in HEK_{CTRL} cells (**P* < 0.05 at 75nM and *****P* < 0.0001 from 100 nM ATV) (Fig. 3.3A).

Moreover, the expression of one of the two variant proteins in the HEK_{OATP2B1var-MRP1WT} or HEK_{OATP2B1WT-MRP1var} significantly reduced intracellular ATV accumulation compared with that in the WT double transfectant model HEK_{OATP2B1WT-MRP1WT} (***P* < 0.01 at 75nM, ****P* = 0.001 at 100nM and *****P* < 0.0001 from 150nM for HEK_{OATP2B1var-MRP1WT} and #####*P* < 0.0001 from 100 nM ATV for HEK_{OATP2B1WT-MRP1var}) (Fig. 3.3B).

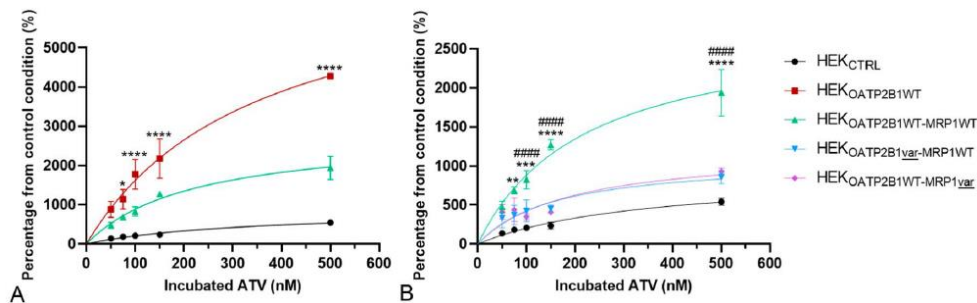


Figure 3.3. ATV accumulation experiment represented in percentage of accumulation from the control condition (ATV accumulation in HEK_{CTRL} at the lowest concentration) in (A) single

transfectant HEK_{OATP2B1WT} versus double transfectant HEK_{OATP2B1WTMRP1WT} and versus HEK_{CTRL} (*P < 0.05, ****P < 0.0001 compared with double transfectant WT model) and in (B) double WT transfectant versus variant models (**P < 0.01, ***P < 0.001, ****P < 0.0001 compared with HEK_{OATP2B1var}-MRP1WT model and #####P < 0.0001 compared with HEK_{OATPWT}-MRP1_{var}).

Effect of *ABCC1*_{2012G/T} and *SLCO2B1*_{935G/A} co-expression on atorvastatin efflux

As expected, HEK_{CTRL} cells showed a weaker efflux ratio compared with the three tested double transfected cell lines (Fig. 3.4). After 60 minutes of efflux time, a non-significant trend ($P > 0.05$) of a higher efflux ratio for the double transfectant model HEK_{OATP2B1WT}-MRP1_{var} was observed compared to cell lines expressing WT MRP1 (HEK_{OATP2B1WT}-MRP1WT and HEK_{OATP2B1var}-MRP1WT). Both cell lines expressing MRP1WT were characterized by an intermediate efflux ratio. Interestingly, the OATP2B1 variant did not affect the efflux ratio. This observation is consistent with the fact that OATP2B1 is an influx transporter and is thus expected to affect accumulation after 120 minutes rather than on drug efflux.

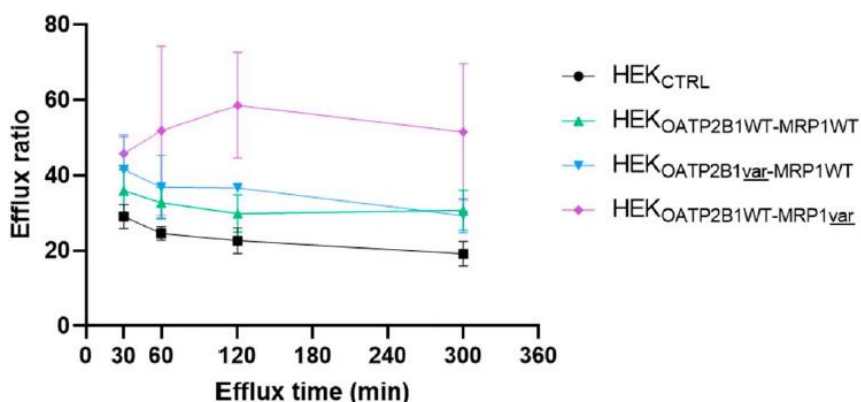


Figure 3.4. ATV efflux ratio in cell models from 30 to 300 minutes of efflux time. The efflux ratio is reporting the effluxed ATV amount in the media compared with the initial intracellular ATV amount, normalized on protein content. Non-significant results, $P > 0.05$ with Bonferroni post-hoc tests.

5. DISCUSSION

Increasing evidence supports the involvement of transporters in the biodistribution of drugs. However, little information is available on the local cellular consequences of their expression and on the functional relevance of SNPs in explaining differences in intracellular drug accumulation. In this study, we generated different recombinant models overexpressing proteins that are assumed to play an important role in the local PK of ATV. In fact, OATP2B1 and MRP1 are both expressed at the skeletal muscle tissue membrane where ATV dose-dependent toxicity is exerted [12]. Going one step further, we introduced SNPs in the coding sequences of both transporters and created single and double transfectant models to measure the relative importance of their respective activities and evaluate the importance of common genetic polymorphisms.

The principal observations that arose from our investigations are likely to be of importance because we (i) confirmed the implication of both OATP2B1 influx and MRP1 efflux transporters in the cellular disposition of ATV, (ii) demonstrated that MRP1 efflux activity counteracts the ATV influx generated by OATP2B1, and (iii) showed that both investigated SNPs rs12422149 and rs45511401 affect the transport activity towards ATV, with rs12422149 being associated with a lower OATP2B1 influx and, rs45511401 with a higher MRP1 efflux.

In single transfectant models, we showed that OATP2B1 overexpression increased intracellular ATV accumulation, confirming its implication in ATV cellular trafficking. We also observed that rs12422149 SNP decreased this boosting effect, suggesting a defective effect on its activity. Second, our results in double transfectant models demonstrated that MRP1 efflux activity reduces the increased accumulation generated through OATP2B1 influx and more effectively when the rs45511401 SNP was introduced in *ABCC1* coding sequence. These observations suggest that the efflux activity of MRP1 seems to be boosted by the SNP because for a comparable OATP2B1 activity (OATP2B1WT), the accumulation was lower in HEK_{OATP2B1WT-MRP1var} than in HEK_{OATP2B1WT-MRP1WT}. Efflux kinetics experiments corroborated the results of accumulation experiments and consolidated our assumption of increased activity

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of the *ABCC1* variant (rs45511401). By contrast, the SNP introduced in *SLCO2B1* coding sequence seemed to decrease the OATP2B1 influx activity as for a comparable MRP1 activity (MRP1WT), the accumulation in HEK_{OATP2B1var}-MRP1WT was lower than that in HEK_{OATP2B1WT}-MRP1WT. This observation also confirmed the results obtained in the single transfectant model with HEK_{OATP2B1WT} and HEK_{OATP2B1var}. A limitation of the efflux ratio is that the ATV extracellular content at the different time points is divided by the intracellular content at the start of the experiment. Meaning that the normalization is performed on cells issued from different wells.

The observation that ATV is being transported by the influx protein OATP2B1 is in accordance with the study of Grube *et al.* [24]. Concerning the effect of the rs12422149 *SLCO2B1* SNP, to the best of our knowledge, this is the first study evaluating the effect of this SNP on the activity of the transporter toward ATV either *in vitro* or *in vivo*. When considering other statins, in a mixed cohort of 34 healthy individuals and 40 patients, Tsamandouras *et al.* showed that the rs12422149 SNP in *SLCO2B1* was associated with an increased apparent simvastatin acid clearance using population PK modeling [20]. The authors concluded that this missense polymorphism may be linked to a lower distribution of simvastatin acid in OATP2B1-expressing tissues, explained by a decreased OATP2B1 influx leading to a paradoxically higher elimination rate. Along the same lines, Kim *et al.* highlighted in 2017 that among 18 volunteers, carriers of the rs12422149 *SLCO2B1* SNP, rosuvastatin was less effective at reducing LDL-C after 8 weeks of treatment ($P = 0.012$) [21]. This could be explained by reduced OATP2B1 influx transport of the drug into hepatocytes, leading to reduced efficacy of rosuvastatin and increased levels of the drug in the blood circulation. It is important to stress that OATP2B1 has higher expression levels in the liver than in the muscle and is also expressed at lower levels in the lungs and placenta. However, to the best of our knowledge, OATP2B1 is the only ATV influx transporter expressed in skeletal muscle tissue. Considering the large tissue distribution, the consequences of this SNP at the systemic level are difficult to predict. On the one hand, a decreased distribution of the drug in peripheral tissues where the protein is expressed (e.g. the skeletal muscle and the lungs) is expected. On the other hand, the decreased OATP2B1 influx activity in

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hepatocytes would reduce the hepatic uptake and, consequently, the active fraction along with drug clearance, leading to a higher systemic exposure in all cases [4]. However, it is important to stress that in the hepatic tissue, other influx transporters known to be implicated in the hepatic uptake of statins, such as OATP1B1 or 1B3, can eventually compensate for the decreased OATP2B1 activity. As a consequence, and given the lack of OATP1B1 and OATP1B3 expression in the skeletal muscle tissue, one can speculate that the functional defect caused by this SNP would have a greater impact on local exposure (i.e. in the myocytes) where no other influx transporters can compensate for the consequent decreased influx activity, than on the systemic clearance, where other transporters can compensate for this decreased activity [25]. Therefore, we showed that in an isolated model of OATP2B1 overexpression, this SNP was associated with reduced cellular influx of ATV, suggesting a theoretical protective effect of this SNPs on the risk of atorvastatin cellular accumulation and possibly muscle symptoms.

We also highlighted that the rs45511401 SNP (c.2012G>T) in *ABCC1* was associated with an apparent increase in efflux activity of MRP1 against ATV using our double transfectant models, suggesting that this SNP could protect against ATV intracellular accumulation. By contrast, in the single transfectant MRP1 model, no effect of MRP1 efflux on ATV accumulation was observed, except at high concentrations (see Supplemental Data, Figure S3.2, Supplemental Digital Content 1, <http://links.lww.com/TDM/A619>). This might be because the affinity (Km) of the transporter is too low and requires prior activity of an influx of drugs to assess its implication in drug transportation. This observation agrees with the *in vitro* study of Knauer *et al.*, which showed that the impact of MRP1 activity is significant only when co-expressed with the rodent-Oatp2b1 [12]. Few data are available regarding the impact of this *ABCC1* SNP on ATV transport. Nevertheless, in 2017, Behdad *et al.* reported a study of 179 patients with primary hypercholesterolemia related to the impact of rs45511401 in *ABCC1* and rs1045642 in *ABCB1* on clinical response to ATV. The investigation did not reveal any significant difference in lipid-lowering response when comparing patients expressing the *ABCC1* wild-type or variant genotype [26]. As for OATP2B1, the lack of a significant effect on systemic exposure and on the LDL-C lowering effect does not exclude a

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local PK effect because the protein is more importantly expressed in peripheral tissues, especially in the skeletal muscle tissue. Transcriptomic data revealed an *ABCC1* relative mRNA expression level of 1.552 transcripts per million in the liver, whereas levels were 10-fold higher in the skeletal muscle (15.65 transcripts per million), arguing in the sense of a pronounced local rather than a systemic effect (GTEx Portal) [27]. To the best of our knowledge, no study has evaluated the impact of both SNPs, either in *ABCC1* or in *SLCO2B1* on the susceptibility to ATV-related muscle side effects.

Although this study helps to clarify the impact of transporter overexpression on ATV cellular accumulation and the impact of common SNPs in an isolated cellular HEK293 system, it does not consider (1) the impact of transporters at their physiological level of expression and (2) the impact of the expression of other transporters in a multicompartmental system (ie, the body). In fact, even if, among the transporters implicated in ATV PK, OATP2B1 and MRP1 are the most expressed in the muscle tissue, it does not exclude that the activity of other transporters affecting systemic PK could have an indirect effect on muscle local exposure. It is well known that other transporters, especially OATP1B1 and OATP1B3, can drive the transport of ATV and are predominant in ruling the systemic PK through hepatic uptake, highlighting that the physiological situation is much more complex than our *in vitro* model. Furthermore, similar experiments with ATV lactone are warranted, as even if controversial, some studies have reported lactone-associated muscle toxicity [28].

6. SUPPLEMENTAL DATA

Generation of *SLCO2B1*_{935G/A} and *ABCC1*_{2012G/T} plasmids

Plasmids sequences were verified using Sanger method (Eurofins, Germany) represented on Figure S3.1.

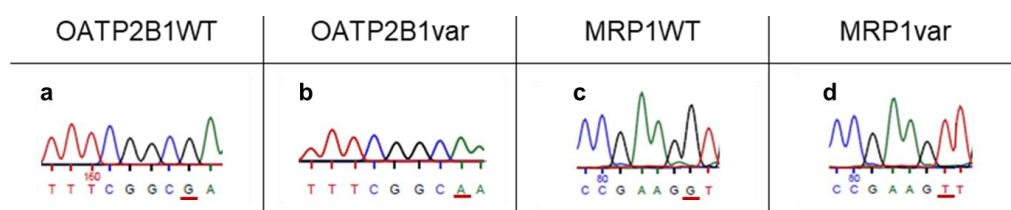


Figure S3.1. Gene sequencing of HEK293 transfected cells with (a) OATP2B1WT, (b) OATP2B1var, (c) MRP1WT and (d) MRP1var.

Generation of stable *ABCC1*_{2012G} and *ABCC1*_{2012T} overexpressing cell lines and characterization of OATP2B1 expression level

Models were created using the same method as for the creation of *SLCO2B1*_{935G} or *SLCO2B1*_{935A} models. Cells were selected in culture with gentamicin (G418) in culture and overexpression of MRP1 protein was validated by FACS.

Effect of *ABCC1*_{2012G} versus *ABCC1*_{2012T} overexpression on ATV intracellular accumulation

Results presented in Figure S3.2 compare the intracellular ATV accumulation between HEK_{MRP1WT} and HEK_{CTRL}. ATV accumulated after 2h of incubation is lower in HEK_{MRP1WT} compared to HEK_{CTRL} at every concentration although significant at 150 and 500nM of the drug (***P* < 0.0001 at both concentrations).

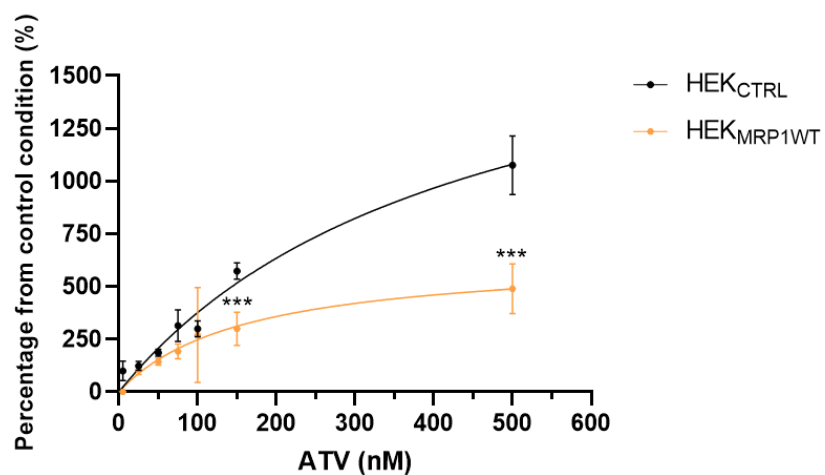


Figure S3.2. ATV accumulation experiment represented in percentage from the control condition (ATV accumulation in HEK_{CTRL} at the lowest concentration) in single transfectant model HEK_{MRP1WT} compared to the control cells HEK_{CTRL}. (***) $P < 0.0001$ HEK_{MRP1WT} compared to HEK_{CTRL}.

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CHAPTER IV - TRANSLATIONAL INVESTIGATIONS

This chapter is adapted from our research article by Hoste *et al.*, entitled **“Silencing drug transporters in human primary muscle cells modulates atorvastatin pharmacokinetics”**, that was submitted for publication in *British Journal of Pharmacology* in January 2024.

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Silencing drug transporters in human primary muscle cells modulates atorvastatin pharmacokinetics

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Data availability statement

All datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors have no conflicts of interest.

Authors contribution

E.H., V.H. and L.E. designed the research; L.P. provided and initiated the myotube culture; E.H. and N.P. conducted the experiments; R.T, A.P. and G.G.M. performed the HPLC/MS-MS analysis; M.L. and S.P.d.R performed the proteomics analysis; E.H., V.H., and L.E. drafted the manuscript; all authors reviewed and approved the final version of the document.

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Abbreviations: AA, antibiotic-antimycotic; ATV, atorvastatin; CFDA, 5[6]-carboxyfluorescein diacetate succinimidyl ester; CV, coefficient of variation; DM, differentiation medium; PK, pharmacokinetics; PM, proliferation medium; SNP, single nucleotide polymorphism; SRM, statin-related myotoxicities.

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What is already known?

- ATV lactone to ATV acid ratio in muscle tissue is correlated with the risk of developing statin-induced muscle side effects.
- ATV cell accumulation and systemic PK is modulated by influx and efflux transporters.

What this study adds?

- The expression of drug transporters in differentiated myotubes is highly variable among individuals and the expression is higher for efflux compared to influx transporters.
- Silencing *ABCC1* or *ABCG2* efflux transporter expression drastically alters ATV myotube accumulation whereas silencing influx transporters has only moderate impact.

Clinical significance?

- The concomitant use of *ABCC1* or *ABCG2* inhibitors with ATV could potentialize the risk of ATV-induced side effects through ATV muscle accumulation modulation.
- Pharmacogenetics and/or cooperation between influx and efflux transporters might modulate the effect of transporter inhibition.

Keywords

Inhibitors, inter-individual variability, influx transporter, efflux transporter, primary human skeletal muscle cell cultures.

1. ABSTRACT

Background and Purpose: Non-adherence to Atorvastatin (ATV) treatment is relatively common and partly due to statin-related myotoxicities (SRM). The risk of developing SRM is dose and concentration-dependent, highlighting the importance of ATV pharmacokinetics. This study explores the inter-individual variabilities in ATV transporter gene expression contributing to ATV modulation within the muscle cell.

Experimental Approach: mRNA levels of efflux and influx transporters were measured and modulated with siRNAs to evaluate the impact on ATV intracellular accumulation in primary cultures of differentiated myotubes from twelve volunteers.

Key Results: All genes assessed are expressed with a high inter-individual variability. In differentiated myotubes, efflux transporters are expressed at higher levels when compared to influx carriers. When considering efflux and influx transporters separately, *ABCC1* and *SLCO2B1* are the efflux and influx transporters the most expressed. Among all genes assessed, *ABCC1* expression was the only one found to correlate with intracellular ATV accumulation. Repression of *ABCC1* and *ABCG2* mRNA levels with siRNA significantly increased the ATV intracellular accumulation in differentiated myotubes. Interestingly, the siRNA targeting *ABCG2* had moderate impact on ATV intracellular accumulation in a volunteer expressing the *ABCG2* variant rs2231142 (c.421C>A, p.Gln141Lys). Reduction of *SLCO1B1* and *SLCO2B1* mRNA levels had a significant impact on ATV intracellular accumulation only in some volunteers, depending on the expression levels of transporters.

Conclusions and Implications: Inter-individual variability in *ABCC1* expression correlates with ATV intracellular accumulation. Silencing *ABCC1* or *ABCG2* expression drastically alters ATV myotube accumulation whereas the effect of silencing influx transporters depends on the expression levels of transporters.

2. INTRODUCTION

Atorvastatin (ATV) has proven its high efficacy to lower the risk of cardiovascular diseases development via the reduction of high cholesterol blood levels [1]. However, the number of non-adherent patients can vary from 24% in secondary prevention to 43% in primary prevention within 24 months of treatment and even increase to up to 56% within a 12-year time window [2, 3]. The main cause of discontinuation is the occurrence of muscle side effects [4]. Diverse mechanisms of ATV-related muscle toxicity have been highlighted but remain incompletely clarified. As an HMG-CoA reductase inhibitor, ATV competitively inhibits the mevalonate pathway implicated in cholesterol synthesis [5]. Behind its therapeutic activity, ATV mediated-HMG-CoA reductase inhibition also accounts for its adverse effects, among which muscular complaints. Indeed, the ATV-mediated inhibition of the mevalonate pathway, and the consequent downstream metabolite depletion, leads to a joint reduction of their respective physiological activity that might constitute one of the possible explanations for ATV-related muscular side effects [6]. ATV-mediated HMG-CoA reductase inhibition and the resulting metabolite depletion is known to be dose- and plasma concentrations-dependent [7]. This suggests that understanding the ATV intracellular pharmacokinetics (PK) is essential to unravel the reasons for the variabilities in treatment response and in the susceptibility to muscle side effects. In particular, the fact that statin-related myopathy (SRM) is specific to skeletal muscle tissue and is dose- and drug potency-dependent [8-10], provides a rationale to explore the importance of factors affecting intra-myocyte concentrations.

To date, most of the available studies on ATV PK have investigated the systemic effect of specific natural genetic variations on ATV PK profiles and/or singular susceptibility for the development of muscle side effects. Clinical studies have consistently demonstrated that the rs4149056 single nucleotide polymorphism in *SLCO1B1* (c.521T>C, p.Val174Ala) decreases ATV hepatic uptake, thus associates with high ATV systemic exposure and increases the risk of muscular side-effects. [2, 11-13]. Interestingly, studies using recombinant cell models have also demonstrated that *ABCC1* and *SLCO2B1* might play an important role in explaining the modulation of ATV accumulation at the cellular level [14, 15], while a case-control pharmacogenetic study highlighted a potential role of the *ABCG2*

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variant rs2231142 (c.421C>A, p.Gln141Lys) in explaining a part of the susceptibility to develop muscle discomfort in patients [16]. Investigating how influx and efflux transporters modulate ATV intracellular accumulation in primary cultures of skeletal muscle tissue, based on the differential expression levels of the transporters, might provide new insights into understanding this issue. This is particularly relevant since it has been demonstrated that the ATV lactone to ATV acid ratio in muscle tissue is correlated with the risk of developing statin-induced muscle side effect [17, 18]. As ATV lactone is more lipophilic compared to the acid form, it easily crosses biological membranes by passive diffusion, whereas the distribution of the acid form is thought to be modulated by active influx and efflux transporters [19]. Hence, understanding the mechanisms of ATV acid accumulation within the muscle cell appears to be one of the key components.

Altogether, data reported in the literature provides evidence of the implication of influx and efflux transporters in ATV systemic PK. However, scarce data are available with regards to their expression in the muscle tissue and their specific contribution to local ATV accumulation within the muscle cells. Our study will thus explore the relative expression of influx (*SLCO1B1*, *SLCO1B3* and *SLCO2B1*) and efflux (*ABCC1*, *ABCC2*, *ABCC4* and *ABCG2*) ATV-transporters in primary differentiated human myotubes and the consequences of transporter-specific expression modulations, taking into consideration selected genetic variations, on ATV intracellular accumulation. Knowing the individual factors explaining the singular susceptibility for developing muscle discomfort could assist the clinician in anticipating potential compliance problems with ATV therapy with dosing adaptation or alternative therapeutic strategies.

3. METHODS

3.1 Chemicals

ATV calcium salt and d5-ATV calcium salt were both purchased from Alsachim (Illkirch Graffenstaden, France). MK-571 sodium salt hydrate and 5[6]-carboxyfluorescein diacetate succinimidyl ester (CFDA) were all purchased from Sigma-Aldrich (St.-Louis, MO).

3.2 Human primary skeletal muscle cell culture

The Hospitalo-Facultaire Saint-Luc – UCLouvain ethical committee approved the protocol under the reference 2020/06OCT/488. All volunteers provided informed consent before taking part to the study. Human primary differentiated myotubes cultures used in the present study were derived from vastus lateralis muscle biopsies from twelve men volunteers (age: 29 ± 9 y, BMI: 27.2 ± 4.1 kg·m⁻²). Collected as described previously [20].

The biopsies were cryopreserved at -80°C in a FBS:DMSO mix (90:10, v/v). For the isolation of satellite cells, a part of the muscle biopsy (15-20 mg) was thawed on ice in a 35-mm petri dish (Corning®, NY) in sterile conditions. The biopsy was then cut in small pieces then transferred in a new 35-mm Petri dish and gently covered with a drop of Matrigel® (Corning®) before incubation for 30 minutes at 37°C, 5% of CO₂. Two milliliters of complete media (DMEM high glucose, no pyruvate supplemented with 20% FBS, 0.5% Ultrosor G (Sartorius, Göttingen, Germany) and 4mM of HEPES (Sigma-Aldrich)) were subsequently added to the Petri dish. Once long filaments were observed at the periphery of the explants, the medium was discarded and the explants were detached using 2.5ml of dispase (0.8U/ml)/collagenase (0.1U/ml) (Sigma-Aldrich) for 2h at 37°C. The cell pellet was then washed twice in medium (DMEM high glucose, no pyruvate supplemented with 20% FBS and 1% antibiotic-antimycotic (AA) (ThermoFisher Scientific)) before being resuspended in 10ml of proliferation medium (PM) (DMEM high glucose, no pyruvate supplemented with 20% FBS, 1% AA and 0.5% Ultrosor G) and seeded in a 100-mm Petri dish (Corning®).

After proliferation, the myoblasts were sorted using magnetic-activated cell sorting (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany, Cat# 130-042-501,) with magnetic microbeads directly linked to CD56. For this purification step, the cells were incubated for 30 minutes at 37°C in 4ml of medium (DMEM high glucose, no pyruvate supplemented with

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0,5% BSA (0.22µm filtered)) containing the antibody anti-CD56 diluted 1/1000 (BD Biosciences, Franklin Lakes, NJ, Cat# 559043, RRID:AB_397180). The cells were then detached with cell dissociation buffer (ThermoFisher Scientific) and, after centrifugation, the pellet was washed with 8ml of PBS supplemented with 0.5% BSA and 2mM EDTA. After centrifugation at 200g, the supernatant was discarded and the pellet was resuspended in 80µl of PBS-BSA-EDTA to which 20µl of the secondary antibody Anti-mouse IgG microbeads was added to be diluted 1/5 (Miltenyi Biotec Cat# 130-048-402, RRID:AB_244361) and incubated at 4°C for 15 minutes. The cells were then washed with 8ml of medium and resuspended in 500µl of PBS-BSA-EDTA. For the purification, the labelled cells were added to the sterile LS columns (Miltenyi Biotec, Cat# 130-042-401) of the MACS magnetic support. After three steps of washing, satellite cells retained in the column were mechanically detached using the adapted syringe. The purified myoblasts were eventually seeded in 8ml of PM into a new 100-mm Petri dish.

When needed, cells were regularly split or frozen before reaching full confluency using cell dissociation buffer. Briefly, after 13 minutes of incubation at 37°C with cell dissociation buffer, 3ml of PBS containing 2mM EDTA were added to dissociate the cells, along with 3 ml of PM for cell dissociation buffer neutralization. The entire content was then transferred to a 15ml tube and centrifuged at 200g for 5 minutes. After discarding the supernatant, the cell pellet was resuspended in 10ml of PM and seeded in a new 100-mm Petri dishes to allow proliferation. Cells were then frozen in 1-ml cryotubes composed of FBS and DMSO (90:10, v/v) at -80°C.

Once thawed prior to the experiments, cells were seeded in a 100-mm Petri dish for proliferation and then seeded in 35-mm Petri dishes. At full confluency, the PM was replaced by a differentiation medium (DM) (DMEM high glucose, no pyruvate supplemented with 1% AA and 2% FBS). This medium, low in serum, is known to promote the fusion of myoblasts into a network of myotubes. The experiments were performed on fully differentiated myotubes, seven days after the start of the differentiation.

3.3 qPCR analysis

The total RNA was extracted in 1ml of trizol (TriPure®, Sigma-Aldrich) according to the manufacturer's protocol. The purified RNA was quantified using the EzDrop100 from Blue-Ray Biotech (New Taipei City, Taiwan). The reverse transcription was performed using 1µg of RNA per condition with the kit GoScript Reverse Transcriptase (Promega, Madison, WI) on a BioRad iCycler Thermal Cycler iQ5 Multicolor Real-Time PCR (Hercules, CA) according to manufacturer's instructions. Real-time qPCR analysis was performed in 96-well plates using SsoAdvanced Universal SYBR Green Supermix on the CFX96 Touch™ device and software from BioRad. Accurate quantification of gene copies per sample was achieved by simultaneously running calibration curves and the samples in triplicate, enabling the precise determination of gene copy numbers. Normalization was carried out using the reference gene beta-2-microglobulin (B2M). The purity of the PCR product was confirmed by analyzing the melt curve conducted at the end of the analysis. The detailed sequences of the forward and reverse primers targeting the human genes of interest can be found in Table 4.1 (Eurofins Scientific SE, Ebersberg, Germany).

Genes	Primers forward	Primers reverse
<i>ABCC1</i>	ACTGAATGGCATCACCTTCTC	ACTGAATGGCATCACCTTCTC
<i>ABCC2</i>	CCTCATTGACGACCATCC	ATTCTCAATGCCAGCTTCTT
<i>ABCC4</i>	AAAATGTACGCCTGGGAAAAG	GGTGAAGGTCACAAACACGA
<i>ABCG2</i>	CAGGACTCAATGCAACAGGA	TTCAGGTAGGCAATTGTGAGG
Beta-2-microglobulin (<i>B2M</i>)	ATGAGTATGCCTGCCGTGTGA	GGCATCTTCAAACCTCCATG
Myogenin (<i>MyoG</i>)	ATAACGCGGCCTCTAATTCC	ATCGGGAAGAGACCAGAACA
Myosin (<i>MYH2</i>)	GTGCTGTACAACCTCAAAGAAC	CTCGGGCTTATACACAGGCA
<i>SLCO1B1</i>	ACTGATTCTCGATGGGTTGG	TTCCAGCACATGCAAAGAC
<i>SLCO1B3</i>	AATTTGGACATGCAAGACAATG	AGTGAAAGACCAGGAACACCTC
<i>SLCO2B1</i>	GCTGCCATCCCCTACTTCTT	CTAGGCCATCCTGCTTCTTC

Table 4.1. Forward and reverse primers targeting the human genes of interest.

3.4 Proteomic profiling

On the last day of the myotubes' differentiation, the medium was removed. After centrifugation at 200g, the washed pellet was resuspended in 25µl of lysis buffer. The lysis buffer was composed of sodium deoxycholate (0.5%), triethylammonium bicarbonate (50mM), NaCl (150mM), IGEPAL (CA-630) (1%), SDS (0.1%), and dodecyl maltoside (0.2%) adjusted at a pH between 7.2 and 7.5 and containing the cOmplete™ protease inhibitors cocktail purchased from Roche (Basel, Switzerland). Before digestion, quantification of the total protein content was performed with the use of a Bradford assay purchased from ThermoFisher Scientific, according to the fabricant's protocol. The samples were treated according to the protocol defined in the HYGIEIA project [21].

3.5 Control of the differentiation

The appearance and morphology of the myoblasts and the myotubes was observed and controlled through the bright field microscope Leitz Labovet (Leica Microsystems, Wetzlar, Germany). The pictures were taken with Zeiss AxiomCam MRc (Zeiss, Oberkochen, Germany) at the end of the proliferation and at different stages of the differentiation. In parallel, RNA extraction of the samples was performed as described above, at both the end of the proliferation and of the differentiation. *MyoG* and *MYH2* (see primers in Table 4.1) mRNA levels at the two stages were quantified. The calibration curves for each gene transcript enabled the precise quantification of gene copies in each sample that were further normalized on the B2M expression levels.

3.6 Gene silencing

Silencer™ Pre-Designed siRNAs targeting *ABCC1* (Cat#4390824), *ABCG2* (Cat#4392420), *SLCO2B1* (Cat#AM16708) and *SLCO1B1* (Cat#4392420) as well as the negative control (siRNA scramble Cat#4390843) were purchased from ThermoFisher Scientific. siRNAs were resuspended in nuclease-free water at a concentration of 10µM. The protocol of siRNA transfection has been optimized by testing different timings (and combination of timings) of transfection. As shown in Supplemental Figure S4.1, best gene silencing results were obtained with double siRNA transfection performed at D2 and D4 after myoblasts

differentiation initiation. siRNA transfection was performed with 5µl of Lipofectamine™ RNAiMAX (ThermoFisher Scientific) in 35-mm Petri dishes, following the manufacturer's instructions. The siRNAs were diluted 400-fold to achieve a recommended final concentration of 25nM in the wells.

3.7 Atorvastatin accumulation experiment

On the last day of the differentiation, the media was removed and replaced with 1350µl of fresh DM to which 150µl of ATV was added for a final concentration of 7.5 or 15nM in the Petri dish. In the conditions with the inhibitors, the myotubes were incubated 15 minutes prior to the addition of ATV with MK-571 or CFDA, at final concentrations of 25µM and 20µM, respectively [22]. The cells were incubated for 2h at 37°C, 5% of CO₂ [14]. At the end of the incubation, the Petri dishes were placed on ice, the media was removed and the cells were washed twice with 1ml of ice-cold PBS. ATV was then extracted according to the protocol described in the following section.

3.8 Atorvastatin extraction and quantification

Extraction of ATV contained in the myotubes was performed directly in the 35-mm Petri dishes using 350µl of MeOH:H₂O (60:40, v/v) containing d5-ATV (used as internal standard) at a final concentration of 5nM. The extracted cells were then transferred to an Eppendorf subsequently sonicated for 5 seconds (Vibra-Cell™, Sonics, CT) before being centrifuged at 10500g for 7 minutes at 4°C. Three hundred microliters of the supernatant were ultimately collected into a new 1.5mL tube for the dosage. For protein normalization, 10µl of the supernatant were used to perform Pierce™ BCA protein quantification according to manufacturer instructions. The ATV HPLC-MS/MS quantification was performed on an Acquity UPLC class H couple to Xevo TQ-S (Waters, Milford, MA), as previously described [14].

3.9 Genotyping

Genetic testing on differentiated myotubes was performed using the SOPHiA GENETICS® Community Panel for Pharmacogenomics which is a targeted, capture-based NGS panel,

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including 41 pharmacogenes, developed and tested in collaboration with the RNPGx (French speaking network of pharmacogenetics) according to the instructions of the manufacturer on a MiSeq platform (Illumina®). The laboratory has also an ISO15189 certification for the clinical use of this panel.

In addition, the variants rs45511401 (c.2012G>T, p.Gly671Val) in *ABCC1* (Cat#C__25614373_30) and rs12422149 (c.935G>A, p.Arg312Gln) in *SLCO2B1* (Cat#C__3101331_10) were identified in the volunteers using TaqMan assays (ThermoFisher Scientific). The experiment was performed in 96-well plates, according to the manufacturer's protocol.

3.10 Data and statistical analysis

Graphs and statistical analysis were performed on GraphPad Prism version 9 (La Jolla, CA). Differences between conditions were considered as significant when the *P* value was < 0.05. Paired t-test was used for the comparison of expression levels before and after siRNA treatment and two-way ANOVA with Tuckey post Hoc tests were performed for ATV intracellular accumulation comparisons. All experiments were performed twice in three biological replicates, to the exception of the measurement of ATV variability in the 12 volunteers that was performed only once. Data are reported in mean standard deviation (SD), unless specified. Proteomic analysis was performed on DIA-NN software [23].

4. RESULTS

4.1 Myoblast differentiation

4.1.1 Microscopy

Bright field microscopy images show the evolution from myoblast proliferation (Figure 4.1A, panel (a)) to differentiation into myotubes (Figure 4.1A, panels (b) and (c)). At the proliferation stage (Figure 4.1A, panel (a)) the myoblasts (black arrows) were mononucleated. On the 3rd to 4th day of the differentiation (Figure 4.1A, panel (b)), while single myoblasts were still visible (black arrows), the process of fusion into myotubes had started (red arrow). On the last day of the differentiation (Figure 4.1A, panel (c)), all myoblasts had fused to form a network of myotubes (red arrows). Visual inspection was conducted before initiating any experiment to ensure proper myoblast differentiation.

4.1.2 Expression of the differentiation genes

To further confirm the differentiation of myoblast into myotubes, mRNA expression levels of *MyoG* and of *MYH2* were quantified by RT-qPCR (Figure 4.1B). Whereas *MyoG* is a transcriptional factor promoting genes that are specific to the skeletal muscle, *MYH2* is implicated in the development of functional skeletal muscle fibers [24]. Monitoring their expression at the end of the differentiation of the myotubes is thus complementary and ensures that differentiation occurred. The mRNA expression levels of both *MyoG* and *MYH2* were increased at the end of the process of differentiation when compared to their mRNA expression levels during proliferation (Figure 4.1B). The transcript quantification was performed in each experiment to confirm myotube differentiation.

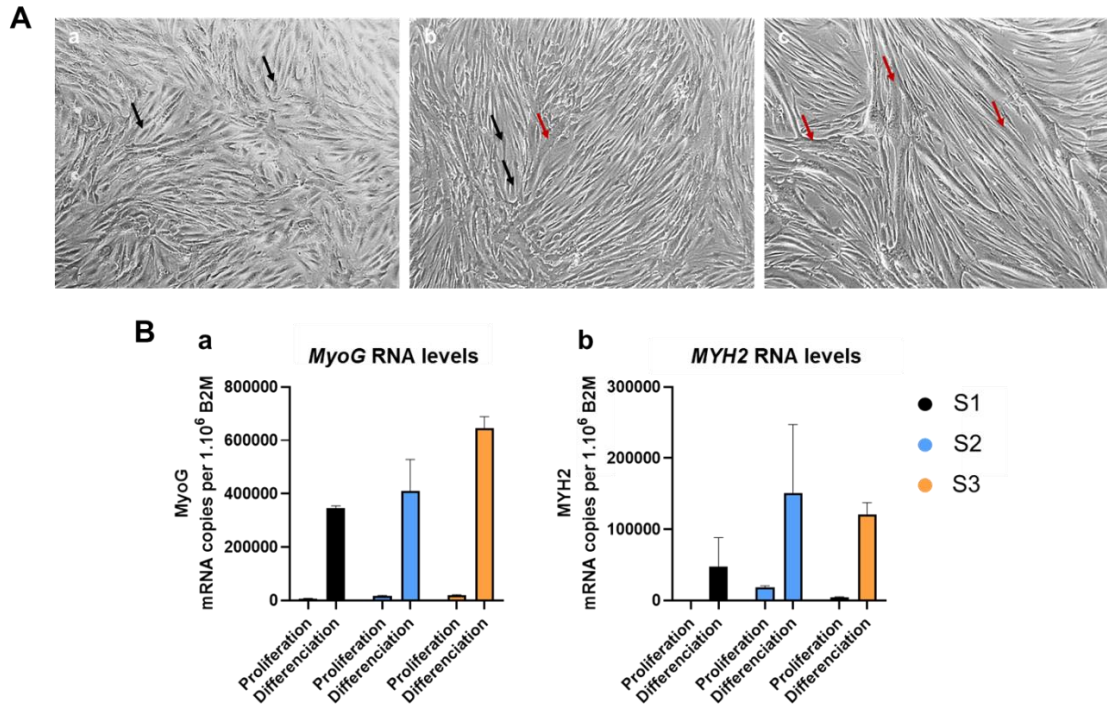


Figure 4.1. Control of the differentiation by (A) bright field microscopy (a) during the proliferation stage, (b) during the differentiation process (D3-D4) and (c) differentiated myotubes on the last day of the differentiation (D7). Black arrows indicate mononucleated myoblasts while red arrows show multinucleated myotubes. Control of the differentiation by (B) RT-qPCR representing the mRNA levels of (a) *MyoG* and of (b) *MYH2* of three volunteers (S1, S2 and S3) at the stage of proliferation and differentiation. The mRNA expression levels are expressed in copies of the gene per 1.10⁶ B2M copies (N=3, n=3).

4.2 Expression of efflux and influx transporters in the skeletal muscle tissue

4.2.1 Transporter expression in differentiated myotubes

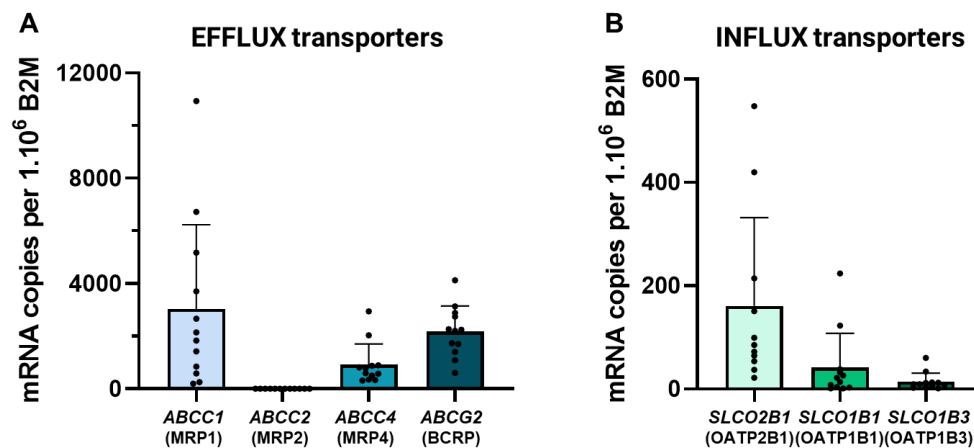
Screening of the mRNA expression levels for the major described efflux (*ABCC1*, *ABCC2*, *ABCC4* and *ABCG2*) and influx (*SLCO2B1*, *SLCO1B1* and *SLCO1B3*) transporters for ATV was performed in the 12 volunteers after myotube differentiation (Figure 4.2). Among efflux

transporters (Figure 4.2A), the mRNA expression levels of *ABCC1* were the highest (mean: 3039.84 ± 3199 copies/ 1.10^6 B2M copies), followed by *ABCG2* (mean: 2179.95 ± 963.6 copies/ 1.10^6 B2M copies) and by *ABCC4* (mean: 916.77 ± 785.8 copies per 1.10^6 B2M copies) whereas *ABCC2* was characterized by the lowest relative mRNA levels (mean: 5.5 ± 1.89 copies per 1.10^6 B2M copies).

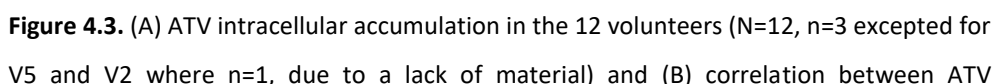
Among influx transporters (Figure 4.2B), *SLCO2B1* showed the highest relative mRNA levels (mean: 160.6 ± 171 copies/ 1.10^6 B2M copies) followed by *SLCO1B1* (mean: 41.3 ± 66.5 copies per 1.10^6 B2M copies). The mean mRNA levels of *SLCO1B3* were the lowest among the influx transporters assessed (mean: 14.3 ± 16.65 copies/ 1.10^6 B2M copies).

For all transporters assessed, a high inter-individual variability in the mRNA expression levels was observed with a coefficient of variation (CV) ranging from 44.2% for *ABCG2* to 160.9% for *SLCO1B1*, with the respective *ABCC1* and *SLCO2B1* CV being 86% and 106.4%.

In parallel, we also assessed the protein expression level by a DIA shotgun proteomics. Only *ABCC1* and *ABCC4* protein were detected in differentiated myotube extracts, probably because the presence of the proteins in the samples is below the detection limit of our system. Nevertheless, our results indicate a significant correlation between mRNA and protein levels for both transporters ($r^2 = 0.6144$ and 0.6483 , $P < 0.05$ for *ABCC1* and *ABCC4*, respectively. See Supplemental Figure S4.2) suggesting that mRNA expression levels reflect well the protein pool of the transporters, at least for *ABCC1* and *ABCC4* that are among the most expressed.



A first screening of the ATV accumulation in differentiated myotubes was performed by incubating the cells with 15nM of ATV. As expected based on the expression of the transporters, a large variability in intracellular ATV levels was observed among the volunteers (CV = 33.91%), with the cells from volunteer V5 being associated with the highest ATV accumulation levels and the cells from V1 with the lowest accumulation levels (Figure 4.3). We next evaluated the correlations between the ATV intracellular levels and the mRNA levels of the different transporters. A significant correlation was observed between the ATV intracellular levels and the mRNA expression levels of *ABCC1* (Figure 4.3b). By contrast, no significant correlation was found with other single efflux or influx transporters (Spearman r ranging from -0.356 to -0.08515 and $P > 0.05$ for *ABCC4* and *SLCO2B1*, respectively).



intracellular accumulation and *ABCC1* mRNA expression levels (r Spearman= -0.5804; $*P = 0.05$).

4.3 Impact of transporter expression modulation on ATV intracellular accumulation

4.3.1 Efflux transporters

4.3.1.1 Volunteer selection and modulation of *ABCC1* and *ABCG2* expression

Whereas *ABCC1* is the most expressed efflux protein in the skeletal muscle tissue, *ABCG2* has been largely studied in the literature for its implication in ATV pharmacokinetics [11, 16, 25, 26]. Furthermore, both genes showed important mRNA expression levels within the differentiated myotubes. For those reasons, we decided to explore mechanistically the impact of both gene expression modulation on ATV intracellular accumulation. For each gene, three selected volunteers (thereafter called S1 to S3) showing either high, intermediate, or low basal mRNA expression levels covering the entire range were selected among the 12 volunteers to perform the gene silencing and ATV accumulation experiments. Data related to all investigated transporters expression in those 3 volunteers are shown in Figure 4.4. Primary myotubes from S2 showed the highest mRNA and protein levels of *ABCC1*, followed by cells from S1 and S3 (Figure 4.5A, panels (a) and (b), respectively) while *ABCG2* was higher in cells from S1, followed by S3 and S2 whose mRNA levels were the lowest among the three selected volunteers (Figure 4.5A, panel (c)). As explained above, *ABCG2* protein levels could not be quantified as they were below the limit of detection of our proteomic method. Both *ABCC1* and *ABCG2* siRNA strongly reduced the mRNA levels when compared to the basal expression (Figure 4.5A, panels (a) and (c)). This drop in mRNA levels was also observed at the protein level for *ABCC1* after treatment of the cells with siRNA (Figure 4.5A, panel (b)).

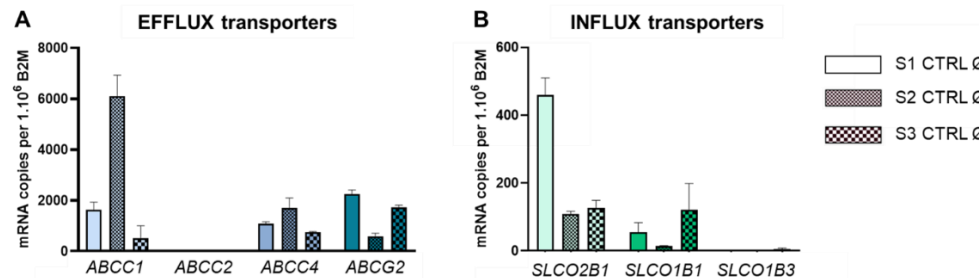


Figure 4.4. Basal mRNA expression of the transporters in control conditions in the volunteers S1, S2 and S3 for (a) the efflux transporters and (b) influx transporters.

4.3.1.2 Impact of *ABCC1* and *ABCG2* gene silencing on ATV intracellular accumulation

Treatment with either *ABCC1* or *ABCG2* siRNA significantly increased ATV intracellular accumulation in differentiated myotubes cultured from the three volunteers' biopsies, when compared to the control condition (Figure 4.5B, panels (a), (b) and (c)). Interestingly, the increase observed in ATV intracellular accumulation after *ABCC1* siRNA treatment was more pronounced in cells from S2, who were characterized by the highest level of *ABCC1* expression, compared to S1 or S3 (389.5% increase for S2 vs 174% $\pm$ 4.55 for S1 and 174% $\pm$ 6.72 increase for S3). Also, the increase in ATV intracellular levels was significantly higher after *ABCC1* siRNA treatment when compared to treatment with siRNA *ABCG2*, especially in S3, where *ABCG2* siRNA treatment had only a minor impact on ATV intracellular levels. Interestingly, S3 was heterozygous carrier of the reduced-of-function *ABCG2* variant rs2231142 (c.421C>A, p.Gln141Lys). However, S2 did also carry the *ABCG2* variant allele and the increase in ATV intracellular concentrations after *ABCG2* treatment was even more important when compared to S1 who was homozygous wild-type for this variant.

Decreasing protein expression using siRNA was compared to protein inhibition using a MRP inhibitor, namely MK-571. In the three volunteers, MK-571 co-incubation significantly increased the ATV intracellular accumulation in comparison to the control condition (Figure 4.5B, panels (a), (b) and (c)). However, MK-571-driven inhibition appeared significantly less important than that observed after both siRNA treatments, except in S3 after *ABCG2* siRNA

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treatment. The impact of influx transporter chemical inhibition through CFDA treatment, a general SLCO inhibitor, was also assessed in the cells from S1-3 (Figure 4.5B, panel (b)). The reduction was however only significant in S2, the highest expressor of *ABCC1* (Figure 4.5A, panel (a)).

Treatment of the myotubes with lipofectamine or siRNA scramble did significantly reduced efflux transporters mRNA levels without impacting the influx transporters mRNA levels (Supplemental Figure S4.3). The reduced expression observed on efflux transporters was however less important than the effect induced by the specific siRNAs. Furthermore, treatment of the cells with lipofectamine alone or transfection with the scramble siRNA had no impact on either *ABCC1* and *ABCC4* peptides levels or ATV intracellular accumulation (Supplemental Figures S4.4 and S4.5). These results indicate that the reduced efflux transporter expression induced by siRNA scramble transfection or lipofectamine treatment is not sufficient to impact on ATV accumulation. Consequently, the impact on ATV intracellular levels resulting from *ABCC1* or *ABCG2* siRNA treatment may primarily be attributed to the specific silencing of *ABC* genes, although it is essential to acknowledge the possibility of off-target effects associated with *ABC* siRNA.

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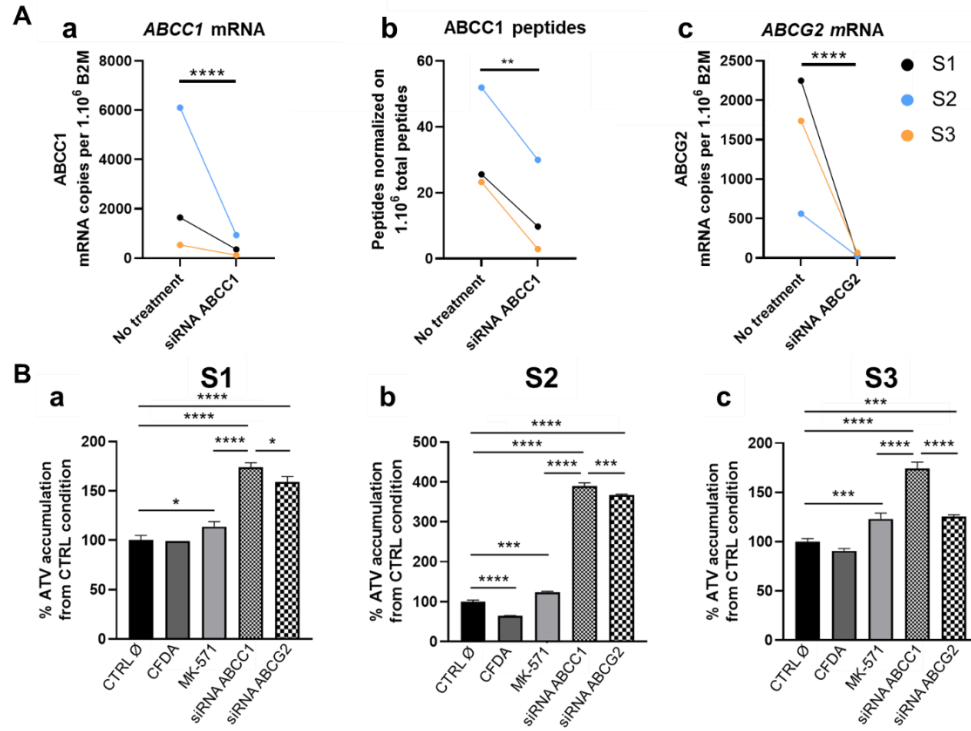


Figure 4.5. (A) mRNA levels of *ABCC1* and *ABCG2* efflux transporters in S1, S2 and S3 differentiated myotubes treated or not with siRNAs; panel (a) *ABCC1* mRNA copies per 1.10^6 B2M, panel (b) *ABCC1* peptides levels and, panel (c) *ABCG2* mRNA copies per 1.10^6 B2M. (B) ATV intracellular levels relative to vehicle-treated cells (CTRL); panel (a) S1, panel (b) S2, panel (c) S3. CTRL $\emptyset$ = no treatment, CFDA = treatment with 5[6]-carboxyfluorescein diacetate succinimidyl ester at $20\mu\text{M}$, MK-571 = treatment with MK-571 at $25\mu\text{M}$. N=3, n=3, repeated twice.

4.3.2 Influx transporters

4.3.2.1 Volunteer selection and modulation of *SLCO1B1* and *SLCO2B1* expression

Among influx transporters, *SLCO2B1* was the most expressed at the mRNA level whereas *SLCO1B1/1B3* were also detected but at low (*SLCO1B1*) and very low (*SLCO1B3*) levels (Figure 4.2B). To assess their implication in ATV intracellular accumulation, gene silencing experiments with specific siRNA transfections were performed to repress either *SLCO1B1* or *SLCO2B1* expression in 4 different volunteers (S4 to S7), *i.e.* 3 per selected genes (Figure 4.7A, panels (a) and (b)). Data related to all investigated transporters expression in the 4 selected volunteers are shown in Figure 4.6. In the control condition, S5, S6 and S7 expressed similar *SLCO1B1* mRNA expression levels. Treatment with *SLCO1B1* siRNA reduced significantly *SLCO1B1* mRNA levels in the volunteers S6 and S7 but not in S5. Considering *SLCO2B1* in control condition, S4 expressed *SLCO2B1* more importantly than S5 and S6 whose *SLCO2B1* mRNA levels were similar (Figure 4.7A, panel (a)). As expected, *SLCO2B1* siRNA treatment significantly reduced *SLCO2B1* mRNA levels in the three selected volunteers. The effect at the protein level could not be assessed as no diagnostic peptide was detected, probably due to the low expression and limited sensitivity of our proteomic method.

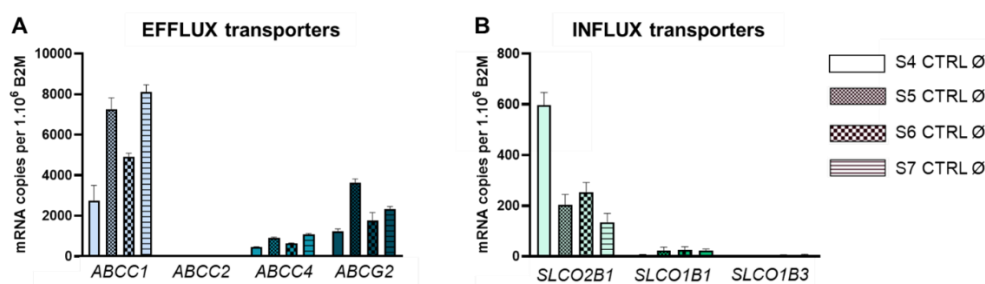


Figure 4.6. Basal mRNA expression of the transporters in control conditions in the volunteers S4, S5, S6 and S7 for (a) the efflux transporters and (b) influx transporters.

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4.3.2.2 Impact of *SLCO1B1* and *SLCO2B1* gene silencing on ATV intracellular accumulation

The impact of *SLCO2B1* gene silencing on ATV intracellular accumulation was measured in the 3 volunteers whereas, for *SLCO1B1*, it was analyzed only in volunteers whose gene silencing was efficient (i.e. S6 and S7). Treatment with *SLCO1B1* siRNA was associated with a significant decrease in ATV intracellular concentrations in S7 only (Figure 4.7A, panel (a)). By contrast, counterintuitively, in S6, treatment with *SLCO1B1* siRNA was associated with a significant increase in ATV intracellular concentrations.

After *SLCO2B1* siRNA treatment, the expected reduced intracellular ATV accumulation was observed in S4, where the expression was the highest in control conditions. Treatment with *SLCO2B1* siRNA in S5 and in S6 resulted in a non-significant increased and unchanged ATV intracellular concentrations when compared to the control condition, respectively.

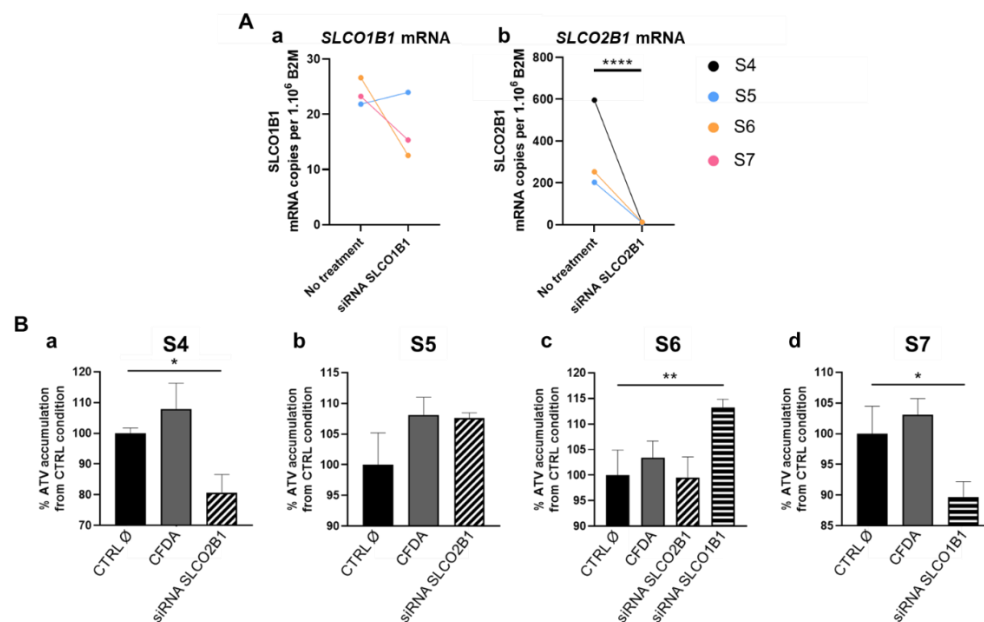


Figure 4.7. (A) influx transporters mRNA levels (as expressed in mRNA copies per 1.10⁶ B2M) of; panel (a): *SLCO1B1* and, panel (b): *SLCO2B1* in S4, S5, S6 and/or S7 differentiated myotubes treated or not with siRNA. (B) ATV intracellular levels relative to vehicle-treated cells (CTRL); panel (a) S4, panel (b) S5, panel (c) S6 and, panel (d) S7. CTRL Ø = no treatment,

CFDA = treatment with 5[6]carboxyfluorescein diacetate succinimidyl ester at 20 μ M. N=3, n=3, repeated twice.

5. DISCUSSION

The importance of ATV transport within myocytes has been poorly investigated to date. Evidence shows that the dynamic of skeletal muscle ATV accumulation might play a crucial role in explaining myotoxic events. Very recently, Lauritzen et al. measured ATV and its metabolites within quadriceps biopsies collected from patients [17]. In this clinical trial, they highlighted an increase in the ratio ATV lactone/ATV acid in the skeletal muscle tissue biopsies of the patients experiencing muscle side-effects compared to those who did not. This observation underscores the significance of ATV transport in explaining muscle toxicities. The lactone form, being highly lipophilic, partly enters and leaves the cell by simple passive diffusion while the acid form, being less lipophilic, mostly relies on active transporters. This suggest that ATV acid transport is relevant to study for explaining modulation of this ratio. In line with that hypothesis, in their study, Lauritzen et al. demonstrated that correlations between plasma and muscle biopsy concentrations were higher for lactone when compared to acid forms of ATV and its metabolites. However, literature lacks reliable expression data of transporters within the muscle tissue and the significance of this expression for modulating ATV transport.

The cohort comprises twelve volunteers, all of whom were men. While it has been demonstrated that the sex does not influence the mRNA expression levels of the transporters in humans [27], the present results could however be biased due to the lack of gender parity. Furthermore, we observed that among the volunteers, three were considered as obese (BMI between 30 and 39.9), six as overweight (BMI between 25 and 29.9) and three as healthy (BMI between 18.5 and 24.9) [28]. It has been demonstrated that obesity is associated with a systemic inflammation, leading to an increase in C-reactive protein (CRP) [29]. Consequently, given that Nies *et al.* associated elevated CRP levels with reduced hepatic *SLCO1B1* mRNA levels, it is plausible to assume that the three obese

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volunteers, might express lower basal levels of the transporters *in vivo* [27]. However, the impact on the expression of *SLCO1B1* on the *in vitro* primary cultures might not be relevant since in our *in vitro* environment cells were culture in the same conditions and the isolated skeletal muscle cells and biopsies were no longer exposed to CRP.

In the present study, we found in our screening of the expression of efflux and influx transporters at the mRNA level, a similar trend in the relative transporter expression when compared to open-source databases (GTEx) [30, 31]. Indeed, our results indicate that *ABCC1* and *SLCO2B1* were the most highly expressed efflux and influx transporters in the differentiated myotubes, respectively. However, the absolute expression levels measured in our study slightly differ from the levels reported in GTEx. For instance, the average *ABCG2* transcript levels were relatively high in our study, i.e. only slightly lower when compared to *ABCC1* mRNA levels while, in the GTEx depository, *ABCG2* levels were close to zero, demonstrating more important *ABCG2* mRNA levels in our volunteers compared to open-source databases of 803 samples. Along the same line, *SLCO1B1* that was not found to be expressed in skeletal muscle tissues in GTEx, was detected at low expression levels in all the volunteers assessed here. Two hypotheses could explain those differences. First, open-source data have been generated directly from muscle biopsies which comprised various cell types, including muscle fibers but not exclusively. In our study, expression data have been obtained from a primary culture of almost pure differentiated myotubes and might potentialize the specificity of the measures and the chance to detect a given gene expression. Secondly, in the GTEx project, mRNA quantification was performed by RNA-seq while, in our study, we used a targeted RT-qPCR method, generally considered as more specific and sensitive compared to RNA-seq. Therefore, the limit of detection for RT-qPCR is believed to be lower than RNA-seq, potentially explaining why *SLCO1B1* was not detected in GTEx but was detected in our study. Overall, our results also indicate a higher level of expression of efflux ABC-transporters when compared to influx SLCO carriers.

Interestingly, not only was *ABCC1* the most expressed transporter gene in differentiated myotubes, but its expression was negatively correlated with ATV intracellular concentrations (Figure 4.3B). The higher is *ABCC1* mRNA levels, the lower the ATV accumulation, consistent with an increased efflux of the drug out of the cell when the

transporter is more highly expressed. Moreover, we found that the mRNA expression levels are a good reflection of the protein content (Supplemental Figure S4.2), at least for *ABCC1* and *ABCC4*, reinforcing the value of this correlation.

This correlation aligns with literature data as it has previously been shown by our group and others that ATV is a substrate for *ABCC1* [14, 15]. However, those studies assessed ATV in recombinant cellular models where the transporter was overexpressed compared to the physiological conditions. Here, our results suggest that, at biological levels of expression, *ABCC1* expression is important to explain differences in ATV cellular transport and accumulation. By contrast, we did not observe any correlation between *ABCG2* mRNA levels and ATV intracellular concentrations despite previous evidence of its involvement in ATV transport [32]. However, in our proteomic survey, we were unable to detect *ABCG2* at the protein level and, it remains still possible that, for *ABCG2*, transcript levels do not reflect the protein content as accurately as for *ABCC1* or *ABCC4*. Notably, as *ABCG2* is a homodimer, the dynamic of translation from transcript to protein may differ [33]. Also, despite ATV is described as a substrate for *SLCO1B1/1B3/2B1*, no correlation was observed between influx transporter expression and ATV intracellular accumulation, possibly because of their lower level of expression and/or the lack of correlation between transcript copy number and protein.

To delve deeper into the causality of the association between transporter expression and ATV intracellular concentration modulations we used molecular (i.e. siRNA) and pharmacological (i.e. transporter inhibitors) approaches. As expected, both the efflux transporter gene silencing and pharmacological inhibition using MK-571 significantly increased ATV intracellular accumulation compared to the control condition. However, although ATV intracellular accumulation significantly increased in the presence of MK-571, the generated increase was substantially less important than with efflux ABC-transporter gene silencing (Figure 4.4B). This observation could be explained by a limited penetration of the chemical inhibitor inside the cells [34].

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Interestingly, when analyzing the interplay of transporter expressions, some of them underscored a significant correlation in their levels of expression (data not shown). Indeed, among the efflux transporters, the expression of *ABCC1* and *ABCC4* appeared to be significantly correlated (**** $P < 0.0001$). This correlation can however not be explained by similar pathways of regulating them since *ABCC1* is being regulated by PXR, CAR and FXR whereas *ABCC4* by ER, AR and PPAR α and located on different chromosomes [35, 36]. Among the influx transporters, the expression of *SLCO1B1* and of *SLCO1B3* was significantly correlated (**** $P < 0.0001$). Both transporters shares > 80% homology and are both, among others, regulated by FXR and HNF [37, 38].

Gene silencing experiments targeting efflux transporters demonstrate that not only *ABCC1* but also *ABCG2* can modulate the intracellular transport of ATV under physiological conditions. We also observed that *ABCC1* siRNA-induced ATV intracellular accumulation was higher than the accumulation observed following *ABCG2* siRNA treatment. However, given its limited amplitude, this difference is likely not relevant for clinical translation. Reinforcing the credibility of the observations, we also noted that the increased accumulation after *ABCC1* silencing was even more pronounced in the volunteer who expressed *ABCC1* most significantly in control condition.

Another potentially interesting observation is that the increase in ATV intracellular accumulation with *ABCG2* silencing was relatively low in the myotubes obtained from S3 compared to S1 and S2 volunteers. Based on the data, we genotyped the volunteers and found that this volunteer is heterozygous carrier of the *ABCG2* variant rs2231142 (c.421C>A, p.Gln141Lys). This suggests that, since *ABCG2* activity towards ATV, rather than *ABCG2* expression, is already reduced [16, 39], silencing the gene expression has only a limited impact on ATV intracellular efflux. However, S2 also carries this variant and the *ABCG2* siRNA-induced increase in ATV intracellular accumulation was almost as high as with the *ABCC1* siRNA. Nonetheless, after siRNA treatment, S2 was characterized by high relative expression levels of *SLCO2B1* (Figure 4) that could potentially have hidden the deleterious effect of this variant. Indeed, one can hypothesize that high drug influx might potentialize the effect of lowering the efflux capacity of the cell. The implication of the *ABCG2* variant

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rs2231142 (c.421C>A, p.Gln141Lys) on ATV transport within the muscle tissue has already been suggested in a case-control study where it was associated with 2.9 greater odds of developing ATV muscle adverse drug reactions [16] without evidence of a clear impact on ATV PK at the systemic level [11, 25, 26, 40, 41]. We believe that the higher expression of *ABCG2* in the skeletal muscle compared to *ABCC2* and *SLCO1B1* might explain that, even with no effect on ATV systemic PK, the local exposure could influence the drug accumulation and by extension, the development of muscle side-effects. In the hepatocyte, *ABCC2*, which is not expressed in the muscle but is 3.5 times more expressed than *ABCG2* in the liver, might compensate for the reduced *ABCG2* expression/activity [42, 43]. Especially since, in the liver, *ABCG2* is about 15 times less expressed than *SLCO1B1* [42, 44]. The reduced hepatic expression of *ABCG2* compared to *SLCO1B1* and *ABCC2* might explain that the effect of the *ABCG2* variant c.421C>A on systemic ATV PK is not always detected. Our study shows, in addition, that the modulation of *ABCG2* activity/expression might have differential impact when considering pharmacogenetic background.

Altogether, these results suggest that, in the clinics, *ABCC1* or *ABCG2* inhibitors might interfere with the local ATV PK and possibly potentialize the muscular side effects. While only few research groups have investigated this hypothesis, the theory seems plausible, as evidenced by a study in rats [45]. In this study, the probenecid (*ABCC1* inhibitor) treated group receiving a statin (rosuvastatin at a dose of 200 mg/kg) exhibited up to 10-fold increase in biological muscle toxicity markers, compared to the groups treated with statin or inhibitor alone [45]. By extension, one could speculate that the concurrent use of *ABCC1* inhibitors (e.g. probenecid, sulfinpyrazone) or *ABCG2* inhibitors (e.g. elacridar, HIV protease inhibitors, tyrosine kinase inhibitors) with ATV might elevate its intracellular accumulation, potentially heightening the risk of myotoxicities in patients [34].

Results of gene silencing experiments are more mitigated when considering influx transporters. Indeed, regarding *SLCO2B1* siRNA treatment, the associated-reduced accumulation was observed in S4 only, whereas no or little impact was observed in S5 and S6, probably because S4 exhibited higher *SLCO2B1* basal expression levels when compared to others (Figure 6). The anticipated reduction in ATV intracellular accumulation with

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SLCO1B1 siRNAs was also only observed in one volunteer (S7). Interestingly, S7 exhibited relatively high expression levels of efflux transporters when compared to S6 (Figure 6), where the treatment with *SLCO1B1* siRNA had a counterintuitive impact (i.e., increased accumulation). This observation raises the question of a potential cooperation between influx and efflux transporters in the transport of ATV in the skeletal muscle myotubes. Our hypothesis is that, when the efflux activity is low, simple passive diffusion might be sufficient for the drug to permeates across the cell membrane. Thus, silencing influx transporter expression has little impact on drug accumulation. By contrast, the role of influx transporter becomes significant when high efflux activity is present in the cell. In such case, a slight (10 to 20%) decrease in ATV accumulation becomes apparent when the influx transporter expression is repressed by gene silencing. This hypothesis of the existence of a cooperation between influx and efflux transporters is somehow corroborated by the observation that, in HEK293 [14] and HSMM [15] transfected cell models, the effect of efflux transporters overexpression on ATV intracellular accumulation was only observed when the *ABCC1* coding sequence was co-transfected with an influx transporter (SLCO2B1 or rOatp1b2).

Surprisingly, our results demonstrate that silencing the expression of influx transporters had less impact on ATV cellular levels than that of efflux transporters. This contrasts with clinical studies evaluating ATV systemic exposure. Indeed, the literature is unanimous on the importance of OATP1B1 activity for ATV hepatic excretion [12, 46, 47]. However, this discrepancy may potentially be explained by the low expression levels of influx transporters in the skeletal muscle compared to the liver, on the one hand, and compared to the expression of efflux transporters in the muscle, on the other. Secondly, at the systemic excretion scale, the activity of OATPs in the liver, in cooperation with ABC transporters, generates a vectorial drug transport from the circulating blood into the bile. The situation might thus be viewed differently when considering a 'static' model in which the drug is effluxed in the same environment where the influx is generated.

Our study has, however, some limitations. First, lipofectamine and the siRNA scramble treatment significantly reduced the expression of the main efflux transporters, compared

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to the control condition with no effect on influx transporters (Supplemental Figures S3 and S4). However, this decrease in efflux transporter expression was less important than the reduction operated with specific siRNAs and had no significant impact on ATV intracellular accumulation (Supplement Figure S5). Secondly, even if the proteomic analysis was sensitive enough to quantify ABCC1 and ABCC4 in our samples, no detection was observed for ABCG2, SLCO2B1 and SLCO1B1. Given the drastic decrease in mRNA levels observed with siRNA treatment, we reasonably assumed that the protein was also decreased.

In conclusion, our study demonstrates that efflux transporters implicated in ATV cellular transport are more expressed in differentiated myotubes than influx transporters that are expressed more moderately. By performing *in vitro* gene silencing in differentiated myotubes and ATV intracellular accumulation experiments in static conditions, we highlight a limited role of influx transporter expression modulation but a major impact of the repression of efflux ABC transporters (mainly *ABCC1* and *ABCG2*) on ATV intracellular accumulation. This second observation could be crucial for explaining drug interactions and statin-related muscle complaints in clinics without an increase in plasma levels. Furthermore, although limited to one observation, our results suggest that pharmacogenetics could confound these results, as c.421C>A *ABCG2* carriers might be less prone to drug-drug interaction involving ABCG2 inhibition.

6. SUPPLEMENTAL DATA

6.1 Optimization of the timing of exposure with siRNAs

Due to the differentiation of the myotubes, the timing of siRNA transfection needs optimization. Seven conditions represented by the different colors, indicate the different timing of exposure with which the siRNAs were added (Figure S4.1). All conditions were assessed at a final siRNA concentration of 25nM, except for the condition 7 (pink), with a final concentration was 37.5nM. Overall, *ABCC1* mRNA expression is reduced in all conditions where siRNAs were added. The condition 3 (orange), however, achieved the most significant *ABCC1* mRNA reduction when the siRNA is transfected two days and four days after the start of the differentiation.

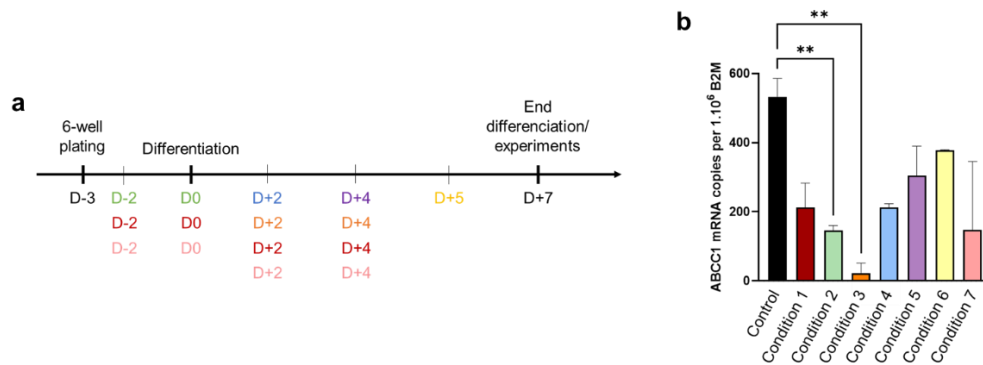


Figure S4.1. Optimization of the timing of exposure with siRNAs in differentiated myotubes. (a) Timeline from the well plating to the end of the differentiation including the conditions tested. (b) Corresponding *ABCC1* mRNA copies per 1.10^6 B2M copies in the 7 tested conditions compared to the control.

6.2 mRNA and protein correlation

mRNA expression levels could be obtained for the four efflux (*ABCC1*, *ABCC2*, *ABCC4* and *ABCG2*) and the three influx (*SLCO1B1*, *SLCO1B3*, *SLCO2B1*) transporters. However, the proteomic profiling only was only able to detect *ABCC1* and *ABCC4*. In the control conditions

(CTRL $\emptyset$), the mRNA levels of *ABCC1* and *ABCC4* correlate significantly to their respective protein levels (Figure S4.2).

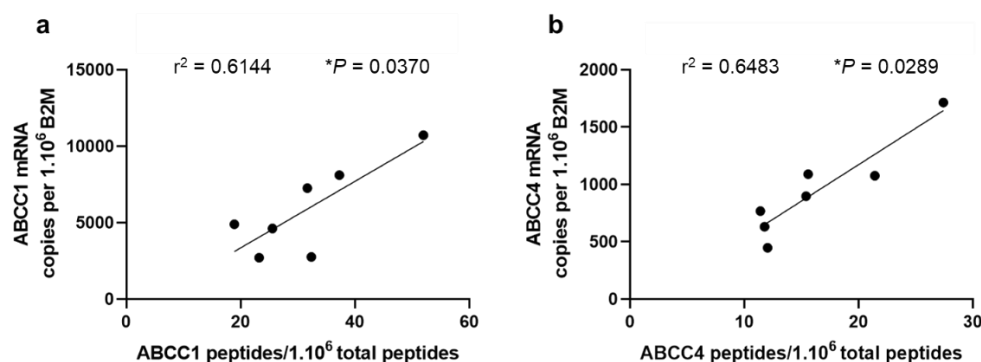


Figure S4.2. mRNA and peptides levels correlation of (a) ABCC1 and (b) ABCC4. Pearson correlation between the mRNA copies of the gene per 1.10^6 B2M copies and the number of peptides of the proteins normalized on 1.10^6 total peptides.

6.3 Impact of lipofectamine and siRNA scramble on transporters' expression and ATV intracellular accumulation

6.3.1 Expression of influx and efflux transporters

6.3.1.1 mRNA expression

mRNA expression levels of efflux and influx transporters have been measured in three control conditions; CTRL $\emptyset$, lipofectamine RNAiMAX and siRNA scramble. The results revealed that the mRNA expression level in both lipofectamine and siRNA scramble conditions of all efflux transporters is significantly decreased, except for *ABCC2* with siRNA scramble which significantly increases its expression (Figure S4.3a). Lipofectamine and siRNA scramble do not significantly affect the mRNA expression levels of the influx transporters compared to the control condition (Figure S4.3b).

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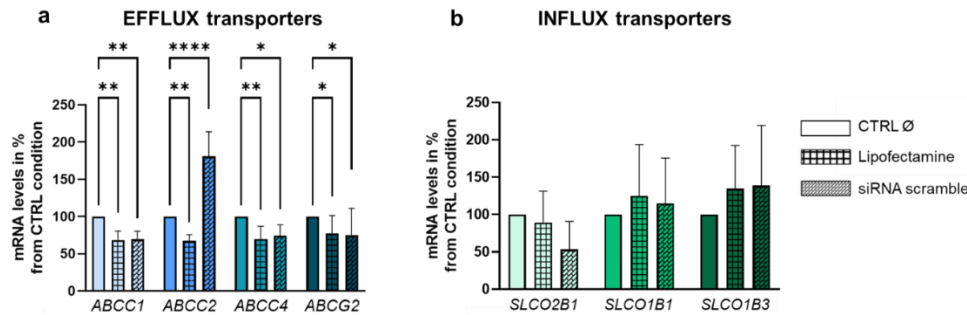


Figure S4.3. Gene expression in CTRL Ø, lipofectamine RNAiMAX and siRNA scramble. Results are represented in percentage of gene expression from the control condition (CTRL Ø) for (a) the efflux transporters (*ABCC1*, *ABCC2*, *ABCC4* and *ABCG2*) and (b) the influx transporters (*SLCO2B1*, *SLCO1B1* and *SLCO1B3*) (* $P < 0.05$, ** $P < 0.01$ and **** $P < 0.0001$). N=7, n=3.

6.3.1.2 Peptides levels

Peptides expression levels of *ABCC1* and *ABCC4* could be measured in the three control conditions. Compared to the control condition (CTRL Ø), neither the lipofectamine nor the siRNA scramble affected the peptides levels of *ABCC1* or *ABCC4* (Figure S4.4).

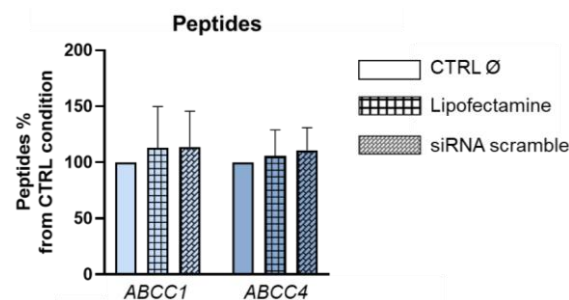


Figure S4.4. Protein expression in CTRL Ø, lipofectamine RNAiMAX and siRNA scramble. Results are represented in percentage of total peptides normalized on the control condition (CTRL Ø). N=7, n=1.

6.4 ATV intracellular accumulation

ATV intracellular accumulation was assessed with lipofectamine RNAiMAX and siRNA scramble and compared to the control conditions (CTRL $\emptyset$) (Figure S4.5). We can observe a high variability in the ATV intracellular accumulation with lipofectamine and siRNA scramble. Compared to the control condition, the ATV intracellular accumulation with the lipofectamine or with the siRNA scramble is non-significantly changed.

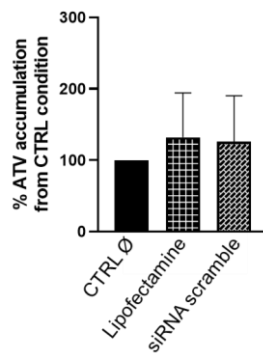


Figure S4.5. ATV intracellular accumulation in three control conditions; myotubes (i) without any addition, (ii) with lipofectamine and (iii) with siRNA scramble. Results are expressed in percentage of ATV accumulation from CTRL $\emptyset$ condition. N=7, n=3.

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CHAPTER V - GENERAL DISCUSSION

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As we will develop in this discussion, both the fundamental and the translational aspects conducted in this thesis are complementary in enhancing our understanding of ATV cellular transport and the potential factors modulating it.

In the fundamental investigation described in Chapter III, we confirmed that ATV is a substrate for the efflux transporter ABCC1 and the influx transporter OATP2B1 in single transfected HEK293 cell models. In double transfectant HEK293 models, we further demonstrated that the increased accumulation caused by OATP2B1 was partially counteracted by the efflux driven by ABCC1. Finally, we associated both single nucleotide polymorphisms; rs45511401 (c.2012G>T, p.Gly671Val) in *ABCC1* and rs12422149 (c.935G>A p.Arg312Gln) in *SLCO2B1*, with a decrease in ATV intracellular accumulation. We showed that both SNPs are protective against ATV intracellular accumulation, while exerting opposite effects on the activity of the respective proteins. The variant in *ABCC1* increases the protein's activity, while the variant in *OATP2B1* reduces its activity, limiting the entry of the drug in the cell.

In the translational part presented in Chapter IV of this thesis, the experiments were performed on primary cultures of myotubes issued from muscle biopsies of twelve volunteers. The screening of the mRNA expression revealed that, among the efflux and the influx transporters, *ABCC1* and *SLCO2B1* were the most importantly expressed, respectively. Moreover, for every gene assessed, a high inter-individual variability in the mRNA expression levels was observed among the volunteers and the expression of *ABCC1* appeared to correlate with the ATV intracellular accumulation. Repressing the mRNA expression of *ABCC1* and *ABCG2* with siRNA significantly increased the ATV intracellular accumulation in the differentiated myotubes. Interestingly, the siRNA targeting *ABCG2* had moderate impact on ATV intracellular accumulation in a volunteer expressing the variant rs2231142 (c.421C>A, p.Gln141Lys) in *ABCG2*. Finally, we demonstrated that the reduction in *SLCO1B1* and *SLCO2B1* mRNA expression had a significant impact on ATV intracellular accumulation only in the volunteers with high basal expression of efflux transporters.

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Limitations of the fundamental investigation and future challenges

The objective of the first part was to evaluate the net impact of the most expressed efflux and influx transporters on ATV intracellular accumulation and the potential implications of their most frequent SNPs. This investigation was conducted on HEK293 transfected cell models. First, HEK293 cells are renal cells and by such, cannot reproduce the specific cellular characteristics of the muscle cells. Secondly, these cells have been genetically modified to overexpress our proteins of interest, which not faithfully reflect the natural expression of these proteins in muscle tissue. Third, technical constrains restricted the number of coding sequences to be transfected together and thus, the number of transporters expressed concomitantly (two transporters maximum). Nevertheless, these technical limitations were by no means a hindrance to responding our hypothesis. HEK293 cells, being embryogenic kidney cells, naturally express very low levels of ABCC1, ABCC4 or ABCG2 (illustrated in black on the Figure 5.1), making them ideal candidates for transfection. The observed ATV intracellular accumulation will then solely reflect the activity of the transfected proteins. As illustrated on the Figure 5.1, the elevation in ABCC1 and SLCO2B1 protein content is evident after transfection of HEK293 cells with plasmids coding for the transporters. It is worth noting that the peptide measurement was conducted more than two years after the cells were last used, which may account for the observed reduction in ABCC1 protein levels compared to SLCO2B1.

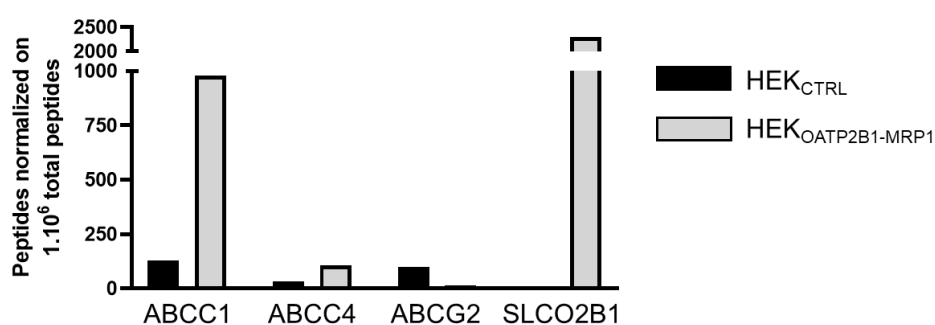


Figure 5.1. Peptide quantification of ABCC1, ABCC4, ABCG2 and SLCO2B1 in non-transfected HEK293 cells in black and in HEK293 cells double-transfected with SLCO2B1 and ABCC1 in grey.

Although we managed to respond to our hypothesis in HEK293 cell models, it would reinforce our results to use an alternative cellular model to confirm that our observations are not dependent on the model used. For instance, Knauer *et al.* used the immortalized cervical cancer cell line (Hela) that also expressed low basal levels of the transporters, making it a good candidate for transfection [1, 2]. Deng *et al.* and Lehtisalo *et al.* on the other hand, used HEK293 cell-derived membrane vesicles to evaluate the effect of ABC transporters on statin transport [3, 4]. These examples represent static models in which influx and efflux transporters facilitate the import and expulsion of substrates within the same environment. To overcome this limitation, transfection of polarized models (MDCKII), as described by Bartholomé *et al.*, would enhance the precision in deciphering the interplay between influx and efflux transporters *in vitro* as this kind of models allows generating vectorial drug flux [5]. However, it might be less relevant for translation to the skeletal muscle tissue situation, as this type of cells is not polarized.

For the ATV intracellular accumulation experiments, HEK293 transfected were incubated with supratherapeutic ATV doses (ranging from 50 to 500nM). This range is higher than ATV plasma concentrations typically observed in patients taking the drug, which in our cohort of ambulatory patients treated with statins ranged from approximately 0.15 to 30nM (Figure 5.2, data extracted from the population published by Stillemans *et al.*), depending on the dose and the time after drug intake [6].

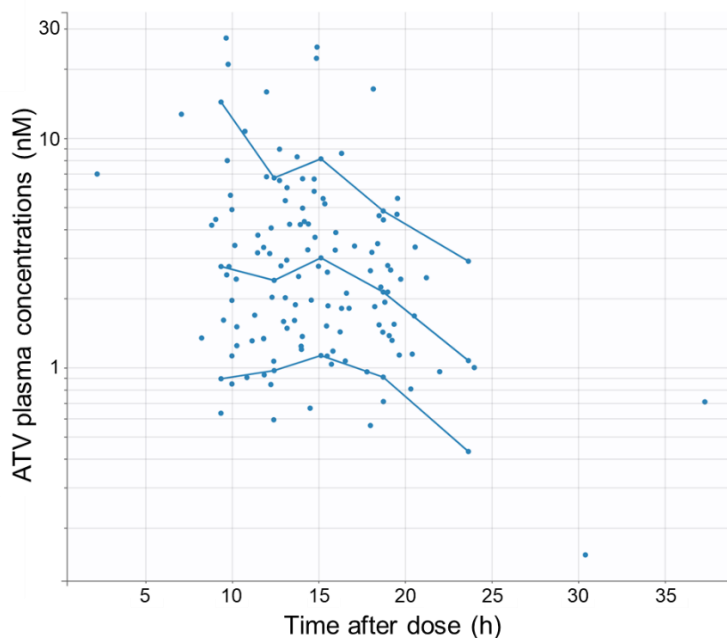


Figure 5.2. Observed ATV plasma concentrations (nM) in a cohort of 70 ambulatory ATV-treated patients (with doses ranging from 5 to 80 mg/day)

Increasing the ATV concentration in experiments with our HEK293 transfected models was necessary due to the overexpression of the transporters and since we wanted to obtain a full spectrum of ATV intracellular accumulation until their saturation. At these supratherapeutic doses, we have performed ATV influx accumulations for two hours and efflux kinetics. It would have been of great interest to conduct both influx and Michaelis-Menten kinetics in our models, allowing us to quantify the effects of the polymorphisms on the affinity (K_m) and/or on the capacity (V_{max}) of the transporters. Indeed, a Michaelis-Menten curve would allow us to detangle whether our SNPs are impacting either on the level of saturation, affinity or the overall catalytic efficiency (i.e. the slope of the curve).

In general, the main limitations of *in vitro* models are the overexpression of limited transporters, designed in a static configuration. Only the general effects observed on ATV intracellular accumulation, whether from wild-type (WT) or variant transporters, remain valid *in vivo* as they reflect their net impact. The direct translation of the other findings to *in vivo* conditions is not feasible with the precited multiple limitations.

Limitations of the translational investigation and future challenges

In the second part of the work, we aimed at using a model that closely mimic the physiological situation, as a logic follow-up of the work conducted on HEK293 models. This biological model allowed us to further evaluate the relative roles and expressions of a broader range of transporters. As described previously, we used primary myotubes isolated *in vitro* and issued from diverse volunteers. This model was adapted to the hypothesis since (i) the cells are muscular in nature, (ii) the transporters are expressed at biological levels and (iii) there is an innate inter-individual variability in their expression. It is still possible, however, that the cells may not express the same levels of the transporters *in vitro* compared to *in vivo*, as we harvest them in an artificial and controlled medium. As well, the differentiation into myotube was also performed in a controlled environment. This *in vitro* setting does not exactly replicate the complex microenvironment of muscular cells, including interactions with other type of cells present in the muscle or surrounding tissues. Nevertheless, considering that this *in vitro* model represents the closest approximation of the reality that we can achieve in a cellular *in vitro* system, even if the expression of the proteins differs from the *in vivo* situation, it is believed adequate to answer our hypothesis. Our ambition was indeed to assess inter-individual variabilities on gene expression and protein activity under the same *in vitro* conditions. If a similar experiment is conducted in the future, it would be worth to consider measuring the expression of transporters on a portion of a fresh biopsy or even more, purify the myotubes directly from this biopsy. With the remaining material, myotube differentiation and myoblast purification could be carried out *in vitro*. Subsequently, by extracting RNA and proceeding to mRNA quantification, we would be able to compare the expression levels of the transporters in our experimental setting with physiological conditions. This approach would enable us to draw conclusions about the real physiological values of mRNA levels in our *in vitro* setting.

For this work, the volunteers were included in the study because of available biological material (opportunistic selection). In the future, we could imagine to conduct a study by prospectively selecting volunteers either based on (i) their genotype if we aim to target the understanding of a specific SNP or (ii) their clinical phenotype and/or previous (in)tolerance

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regards to ATV treatment. Indeed; like in the study of Lauritzen *et al.*, we could consider obtaining biopsies from patients suffering from SRM compared to patients that do not [7]. The originality would be to perform the experiments achieved in this thesis and identify factors influencing the myotoxicity development.

The study and experiments performed on the myotubes *in vitro*, have allowed us to grasp the importance of the efflux transporters, namely ABCC1 and ABCG2, and of the influx transporters, specifically OATP1B1 and OATP2B1, for explaining differences in cellular ATV PK. A cooperation between influx and efflux transporters has been observed in the myotubes, where the *SLCO1B1* and *SLCO2B1* siRNA were effective only in case of high efflux transporter expression. The cooperation was also observed in HEK293 cell models, in which MRP1 efflux significantly reduced ATV intracellular accumulation only when co-expressed with *SLCO2B1*. However, the static state in which the experiments were conducted does not reflect the cooperation of influx and efflux transporters at the systemic scale, where they create a vectorial drug transport in the liver from the blood to the bile. Although the compartments surrounding the skeletal muscle tissue differ from those of the liver, ATV also follows a vectorial drug flow, not represented in our model.

Additionally, the design of our experiments does not consider the variation in ATV plasma concentrations to which the skeletal muscle is subjected *in vivo*. To overcome this limitation, a possibility could be to use a device like a flow-based hollow fiber modeling system represented on Figure 5.3, designed to reproduce the dynamic of plasma concentrations. This device is designed for circulating cells, but we could imagine a similar device tailored for adherent cells.

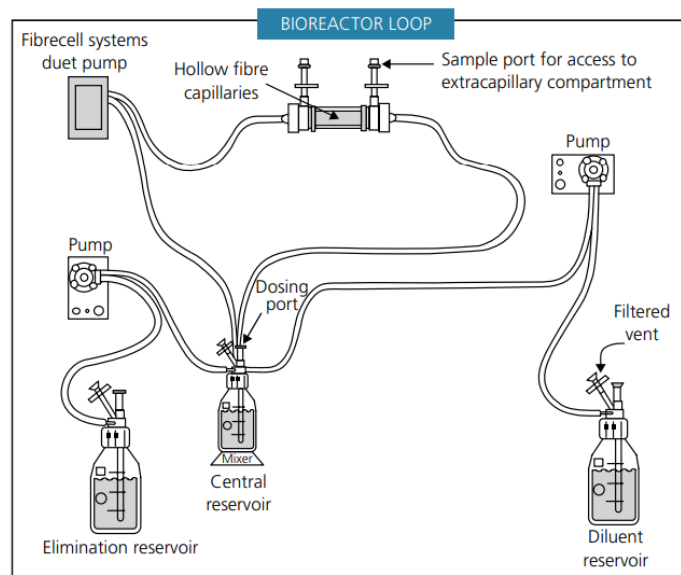


Figure 5.3. Representation of the hollow fiber construct from Cadwell *et al.* for circulating cell contained in the hollow fiber capillaries [8].

Along the same line, an intriguing approach for investigating the dynamic interplay between skeletal muscle and the extracellular compartment involves the application of microdialysis, a technique widely employed in PK research. Microdialysis offers a unique capability to monitor the temporal changes in the composition of the extracellular fluid by employing a semi-permeable membrane that allows the sampling of small molecules from the interstitial space. In the context of our research, microdialysis probes can be strategically placed within the muscle tissue, enabling real-time measure of ATV providing valuable insights into the PK behavior within the skeletal muscle microenvironment [9].

Limitations in the design of the project and future challenges

In the introduction of this thesis work, we highlighted the facts that ATV is the most prescribed statin, in Europe and in the US, as it has been associated with a high efficiency in primary and in secondary prevention [10-13]. However, the drug is also associated with the development of ATV-related muscle toxicity in 7 to 15% of the patients [6, 14]. Many

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putative toxicity mechanisms have been described but the lipophilic nature of the statin influence them. Among the three commercially available lipophilic statins in Belgium, ATV ranks third (Log P partition coefficient (pc) of 1.11), with simvastatin being the most lipophilic (pc of 1.60) (Table 1). Rosuvastatin and pravastatin are considered hydrophilic statins with a negative pc. As lipophilic statins more extensively cross the cell membranes by passive diffusion, they should present higher risk of muscle toxicity than hydrophilic statins. Skottheim *et al.* investigated that point on *in vitro* myotubes, where both simvastatin and fluvastatin acid were significantly more myotoxic than ATV [15]. This underscores the relevance of studying ATV as part of the skeletal muscle transport.

Statins acid forms	Simvastatin	Fluvastatin	Atorvastatin	Rosuvastatin	Pravastatin
Log P (N-octanol/H ₂ O partition coefficient)	1.60	1.27	1.11	-0.33	-0.84
Lipophilicity	Lipophilic	Lipophilic	Lipophilic	Hydrophilic	Hydrophilic

Table 1. Lipophilicity based on their Log P N-octanol/H₂O partition coefficient of the five commercialized statins in Belgium [16].

All together, we acknowledge that replicating the same project with rosuvastatin or pravastatin would be informative. Considering their respective hydrophilicity, we could logically infer that their transport by influx or efflux transporters might be even more significant than for ATV, which partially diffuses passively through the membranes.

Another potential limitation in line with the lipophilicity lies in the fact that, in both parts of the project, we exclusively utilized ATV in its acidic form and not in its lactone form. However, it is known that an elevated ATV lactone/ATV acid ratio in the skeletal muscle tissue is predictive of increased risks of myotoxicities [7, 15]. However, what has not been investigated is whether a high ratio but a low global intracellular ATV content would be more deleterious for the muscle cells than a low ratio but a high global intracellular ATV content. As indicated in the thesis title, the focus of this project was directed towards

understanding the transport of ATV in the skeletal muscle tissue through protein carriers. This is why we opted to conduct the experiments with the acidic form, as it is less lipophilic than its homologous lactone form (which has a Log P_{pc} of 4.24) [17]. In the skeletal muscle tissue, one could speculate that the modulation of the toxicity may involve *ABC* and *SLCO* transporters that would more extensively regulate the ATV acid intracellular levels differently if ATV-lactone is present in the environment (Figure 5.4). It is however difficult to predict what impact this co-incubation will have on the intracellular accumulation and the potential toxicity. It is not yet fully understood to what extent the lactone form is transported by influx and efflux transporters. This uncertainty may impact the transportation of the acidic form, giving rise to a possible competition between the acidic and the lactone form. Such competition may lead to the saturation of transporters, subsequently prompting an increased reliance on passive diffusion. This shift in dynamics has the potential to modulate the ratio between the ATV lactone and acid forms within the skeletal muscle tissue. Adding to the complexity is the unknown interconversion rate between the ATV lactone and its acidic form. This lack of knowledge regarding interconversion rates introduces an additional layer of complexity that could also significantly influence the overall ratio dynamics. This phenomenon is, however, believed to occur spontaneously, rather than through processes involving glucuronosyltransferases and paraoxonases, which are not expressed in the skeletal muscle tissue. To assess the interconversion rate, one plausible approach would be to measure the proportion of ATV acid and lactone in the skeletal muscle tissue but also in the media at the end of the experiment. This dual information could provide valuable insights into a more comprehensive understanding of the dynamics governing the ATV lactone/acid ratio in the skeletal muscle tissue.

To investigate that point, our idea would be to incubate the cells with different ratio of ATV lactone/ATV acid, instead of ATV acid solely, in the present models to better reflect the *in vivo* clinical situation.

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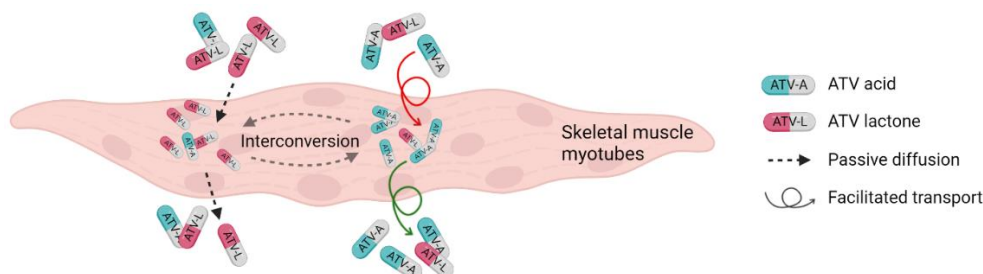


Figure 5.4. Putative transportation interplay of ATV lactone and ATV acid into the skeletal muscle tissue.

In the primary myotubes cultured *in vitro*, we have observed that silencing a given gene might impact on the expression levels of others. As demonstrated on the Figure 5.5a, the mean ABCC4 peptide level increased from 14.2 ± 14.1 to 39.7 ± 16.5 peptides per 1.10^6 total peptides in the control condition when compared to siRNA *ABCC1* treatment. Alike with *ABCC1* siRNA and, in a lesser extent, with *SLCO2B1* siRNA, *ABCC1* and *ABCC4* protein levels were increased (Figure 5.5b). We can also observe that the amplitude of this increase is consistent in a same volunteer. In Figure 5.5a for instance, the volunteer with the lowest *ABCC4* protein content in the control condition is also the one with the lowest *ABCC4* protein levels in the siRNA *ABCC1* condition. This consistency is observed across all conditions. The measurements of *ABCC1* and *ABCC4* peptide levels showed a slight increase with lipofectamine and with siRNA scramble compared to the control condition (CTRL $\emptyset$) (refer to Supplemental data Chapter IV). This phenomenon could potentially explain the elevation of *ABCC1* and *ABCC4* protein content with the siRNA *SLCO2B1*, albeit only partially with the siRNA *ABCC1*. In this case, considering that a lack of specificity of the siRNA *ABCC1* is very unlikely as siRNA specificity was guaranteed by ThermoFisher Scientific, a compensatory mechanism of the transporters may be occurring. We could extrapolate this observation *in vivo* and imagine that patients taking a drug repressing one of the efflux transporters, might show an elevation of other efflux transporters by compensation. Taking it a step further, we might consider that the absence of observed effects of a SNP in a transporter expressed at a basal low level in a tissue might be hidden by, either other

transporters expressed at basal higher levels in the same tissue or by compensation from other transporters for its reduced activity.

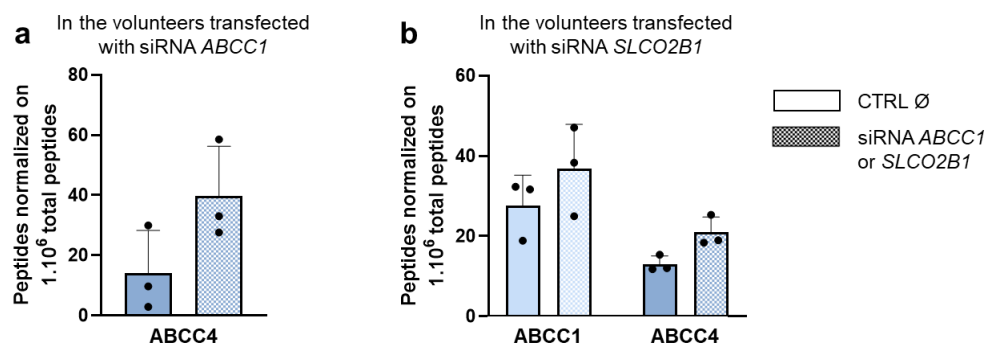


Figure 5.5. Peptide levels of (a) ABCC4 in the three volunteers in control condition (CTRL Ø) and transfected with siRNA *ABCC1* and of (b) ABCC1 and ABCC4 the three volunteers in control condition (CTRL Ø) and transfected with siRNA *SLCO2B1*. Non parametric t test N=3.

Although the utilization of siRNA might appear quite fundamental, extrapolation of the results to the clinic is quite easy. As discussed in the second paper in Chapter IV, the effect induced by the siRNAs *ABCC1* and *ABCG2* could suggest that inhibitors of the transporters could interfere with the local PK and potentiate the development of muscle side-effect *in vivo*. The hypothesis was investigated by Dorajoo *et al.* in rats, where the probenecid (ABCC1 inhibitor) treated group receiving a statin (rosuvastatin at a dose of 200 mg/kg) exhibited up to 10-fold increase in biological muscle toxicity markers, compared to the groups treated with statin or inhibitor alone [18]. By extension, one could speculate that the concurrent use of ABCC1 inhibitors (e.g. probenecid, sulfinpyrazone) or ABCG2 inhibitors (e.g. elacridar, HIV protease inhibitors, tyrosine kinase inhibitors, ticagrelor) with ATV might elevate its intracellular accumulation, potentially heightening the risk of myotoxicities in patients [19]. The interaction between ticagrelor and statins is of particular interest since ticagrelor is an antiplatelet and, both drugs are often taken together after a CV event. A randomized control trial on 24 patients interestingly highlighted an elevation of ATV C_{max} and AUC by 23% and 36% when concomitantly taken with ticagrelor, respectively. An elevation in ATV metabolites was also observed under those conditions. However, there

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was no association found between the elevated ATV plasma concentrations and the development of muscle-related side effects since they were mild (and not muscle-related) and the majority had resolved before the end of the study [20]. The interaction between ticagrelor and other statins was also studied. It was demonstrated that the PK parameters of simvastatin was more extensively increased than those of ATV [20]. The interaction between ticagrelor and rosuvastatin was also observed and even associated with an increased risk of toxicity in patients taking both drugs [21].

For the second part of the thesis led on the differentiated myotubes, we retrospectively genotyped the volunteers. In the future, as mentioned above, we could consider prospectively identifying volunteers with the desired variants and perform targeted experiments. In our random selection, two volunteers were heterozygous for the variant rs2231142 (c.421C>A, p.Gln141Lys) in *ABCG2* and a diminished effect of the siRNA on ATV intracellular accumulation was observed for one of them. In a first time, we could consider selecting a larger cohort of individuals carrying the variant to confirm this single preliminary observation. In a second time, we could imagine to extend it to other variants like the variant c.521T>C in *SLCO1B1* largely studied for its systemic impact, or to the variants c.2012G>T in *ABCC1* and c.935G>A in *SLCO2B1* that were investigated in HEK293 models.

The limited increased intracellular ATV accumulation with the siRNA *ABCG2* in the volunteer carrying the variant c.421C>A in *ABCG2* on ATV intracellular accumulation suggests that the SNP might modulate the effect of a potential interaction. This has been previously observed in other context with other medications. For instance, Belkhir *et al.* demonstrated in a cohort of 135 HIV-1 infected patients that the interaction between darunavir and etravirine, an inducer of the CYP3A5, is only visible in CYP3A5 expressors [22]. Alike the studied variant in *ABCG2* that decreases the transporter activity, the *CYP3A5*3* allele induces a loss-of-function of the enzyme. Interestingly, they observed the inducing effect of etravirine on *CYP3A5* darunavir metabolism only in CYP3A5 expressors, i.e. when the enzyme was functional. Similarly, our observation suggests that *ABCG2* repression is only relevant when the transporter is not affected by the SNP. Both observations suggest that the pharmacogenetics can modulate an established drug-drug interaction.

In clinical settings, the literature highlights the implication OATP1B1 on ATV systemic clearance. ATV clearance is reduced in patients carrying the deleterious SNP rs4149056 (c.521T>C, p.Val174Ala) in the influx transporter. To a lesser extent, in our popPK study, reduced ATV apparent clearance was observed in patients taking L-thyroxine, an inhibitor of OATP2B1 [6]. The reduced ATV systemic clearance conducts to less ATV entering the liver and thus reaching its therapeutic target, leading to a diminished lowering cholesterol response. The reduced clearance is also associated with increased ATV systemic exposure and this increased exposure is further correlated with increased risks of SRM [23]. In our PopPK study, we demonstrated a negative correlation between ATV apparent clearance and creatine kinase (CK) serum levels (Figure 5.6).

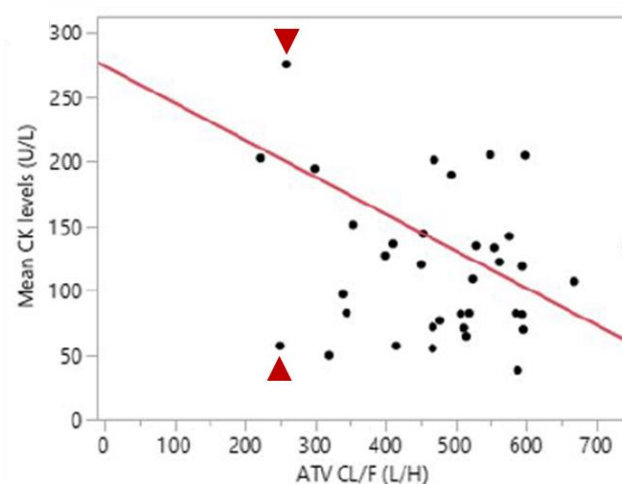


Figure 5.6. Correlation between ATV clearance and creatine kinase serum levels from Stillemans *et al.* [6].

While this correlation is statistically significant, we can notice that some of the patients with a low ATV clearance, did not experience myotoxicities whereas patients with high ATV clearance experienced relatively high CK levels. We have also observed that, for a similar ATV clearance, some of the patients exhibited low CK levels, while others, showed high to very high CK levels (an example is pointed by the red arrow on Figure 5.6).

Altogether, the observations made in this work could advance various hypotheses to elucidate the reasons behind these outliers. We could hypothesize that the differential activities of ABC transporters, and to a lesser extent, OATP carriers, within the skeletal

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muscle tissue modulate the ratio of ATV lactone to ATV acid, thereby influencing the development of myotoxicity, independently from the systemic clearance that is primarily dependent on OATP activity in the liver.

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From the laboratory benchtop

Studying the toxicity was beyond the scope of this thesis, which focused on the PK. Nevertheless, it would be intriguing to explore whether the modulation of intracellular accumulation of ATV acid and/or lactone are related to the toxicity markers in our cultures. In this context, we might consider incubating the cells with a ratio of ATV lactone/ATV acid to mimic clinical conditions. Under this condition, we would be able to evaluate the myotoxicity at biological expression levels and compare it with the modulation induced by the siRNAs. Although in a static model, we could also imagine to assess the level of correlation between the intracellular and extracellular compartments as Lauritzen et al. pointed out similar ratios of ATV lactone/ATV acid in the blood and in the skeletal muscle tissue [7]. However, a challenge arises in attempting to achieve a toxic response in skeletal myotubes, given that *in vitro* ATV toxicity assays reported in the literature, utilized significantly higher drug doses. For instance, Knauer et al. conducted toxicity assays with 10 and 100 μ M of ATV. Those concentrations are 2,000 and 20,000-fold higher than the dose we used to incubate the myotubes (5nM) [1]. Similarly, Skottheim et al. performed myotoxicity assays on skeletal muscle cells with 100 μ M to 400 μ M of ATV [15]. The cytotoxicity assays performed in these studies demonstrate a general toxicity of the cells (i.e. ATP-content measurement, formazan formation, caspase 3/7 activation or fluorescein diacetate). While hypothetically considering specific and more sensitive markers of the muscular toxic response such as creatine kinase or intermediary metabolites of the cholesterol pathway, the literature to date lacks substantial information, as even for detecting caspase activation, doses of 10-100 μ M are typically employed.

Given the results obtained in this thesis, we could, in a first time, consider pursuing the investigation on myotubes cultured *in vitro* as our work has generated multiple leads worth to be investigated. First, as enounced in the discussion, we could select volunteers based on their genotype to focus the understanding of a specific SNP. Alternatively, we can select patients based on their phenotype to perform skeletal muscle biopsies and further myotubes investigations to evidence differences between patients that suffer from ATV-related toxicity in comparison to other that do not. Continuing our efforts to closely mimic

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real-life clinical conditions, as explained above, we could also repeat the accumulation experiments by incubating cells with different ratios of ATV acid to ATV lactone.

To the clinical application

In a subsequent phase, or once the results obtained *in vitro* are further confirmed, we could imagine initiating clinical investigations.

First, the objective would be to delve deeper into the interactions between ABC transporters inhibitors and the response to statins. With this in mind, we might retrospectively, or even prospectively, select volunteers who underwent statin treatment concomitantly with inhibitors of ABCC1 or ABCG2 and assess both the PK parameters if available and the potential development of SRM (with a clear definition of SRM). Referenced ABCC1 inhibitors include uricosuric drugs such as probenecid or sulfinpyrazone, commonly used to treat the gout. Due to the short-term nature of this treatment, the likelihood to observe a clinical effect of ABCC1 inhibition on the development of muscle side effects are limited; however, modulation of ATV PK could be observed. On the other hand, known ABCG2 inhibitors include HIV protease inhibitors, tyrosine kinase inhibitors (e.g. erlotinib, nifitinib), ticagrelor (antiplatelet agent), cyclosporine, tacrolimus or angiotensin receptor antagonists used as part of long-term treatments [19, 21, 24, 25]. In this particular patient subgroup, considering the demonstrated impact of ABCG2 inhibitors, specifically ticagrelor on ATV PK, it may be worth exploring the underlying mechanisms behind this interaction. Investigating why the interaction with rosuvastatin is more potent in inducing muscle-related side effects could also provide valuable insights.

To go one step further, we could also evaluate the differences in ATV PK within the patient subgroup mentioned in the previous paragraph, comparing individuals expressing the variant rs2231142 (c.421C>A, p.Gln141Lys) in *ABCG2* with those carrying the WT transporter but this time, also in the muscle tissue and not only at the systemic level. A similar approach might be considered for SNPs located in *ABCC1*.

To conclude

The exploration of ATV-related toxicity dates back to the early 90's, with subsequent extensive literature highlighting various aspects of the problematic. Over the years, numerous complementary mechanisms have been identified, all corroborating the double-edged ATV pleiotropic effects. In parallel, the hypothesis of the PK, intertwined with pharmacogenetics has complemented the intrinsic toxicity mechanisms of ATV toxicity and added a new aspect to their comprehension; the inter-individual variability. The additional insight provided by the systemic modulation of ATV PK via the pharmacogenetics is pivotal. This thesis goes a step further by delving into cellular ATV PK, potentially opening a new chapter where cellular processes finely contribute to elucidating ATV-related toxicities independently of the systemic PK governed by OATPs. It is however with no doubt that these promising results needs to be further confirmed *in vitro* and investigated clinically.

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CHAPTERS V & VI - REFERENCES

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List of tables related to the introduction

SNPs	Frequency	<i>In vitro and/or in vivo effect</i>
rs4149036 (c.359+97C>A)	AFR: 0.507 EAS: 0.527 SAS: 0.163 EUR: 0.223	Retrospective study on 71 renal transplant patients associated the variant with ↑ mycophenolic acid AUC in elder, male and high serum albumin subgroups [1].

Table 7.1. Relevant non-coding SNP in *SLCO1B1*.

SNPs	Frequency	<i>In vitro and/or in vivo effect</i>
rs11045585 (c.1599-5676A>G in 3'UTR of intron 12)	AFR: 0.213 EAS: 0.163 SAS: 0.054 EUR: 0.139	Significantly associated with docetaxel-induced severe leukopenia/neutropenia on a cohort of 84 patients, ↑ their risk by 5.49 [2]

Table 7.2. Relevant non-coding SNP in *SLCO1B3*.

SNPs	Frequency	<i>In vitro and/or in vivo effect</i>	
rs12422149 (3) (c.935G>A, p.Arg312Gln)	AFR: 0.091 EAS: 0.326 SAS: 0.218 EUR: 0.104	See Chapter I – Table 1.7	Associated with a longer time to progression in androgen deprivation therapy in prostate cancer [3], ↑ of 7 months in 935GA and AA [4] Genotype GG (rs12422149), TT (rs1789693) and AG/GG (rs1077858) associated with the shorter time to progression in androgen deprivation therapy [3]
rs1789693 (c.972+3551T>A)	AFR: 0.248 EAS: 0.395 SAS: 0.651 EUR: 0.696	Homo- and heterozygous associated with ↓ mean tissue abiraterone [5]	
rs1077858 (c.1075+2318G>A)	AFR: 0.529 EAS: 0.422 SAS: 0.570 EUR: 0.652	Na	

Table 7.3. Relevant non-coding SNPs in *SLCO2B1*.

SNPs	Frequency	<i>In vitro and/or in vivo effect</i>
rs2235015 (c.287-25G>T)	AFR: 0.355 EAS: 0.056 SAS: 0.159 EUR: 0.194	<i>In vivo</i> data (mice) showed better response to antidepressant drug [6]. No significant effect on 888 patients with depressive disorders [7]
rs2032588 (c.1350+44C>T)	AFR: 0.201 EAS: 0.000 SAS: 0.006 EUR: 0.059	↓ side effects of A allele in Pgp-dependent antidepressant users [8] Associated with 5-fold ↑ antiepileptic drug resistance in males on 86 epileptic patients [9]
rs2235040 (c.2481+24G>A)	AFR: 0.156 EAS: 0.055 SAS: 0.152 EUR: 0.129	Variant significantly associated with selective serotonin reuptake inhibitors (SSRI) adverse drug effects including 2981 participants with mental health issues [10]. Supported by <i>in vivo</i> (mice) data [6]. No significant effect on 888 patients with depressive disorders [7]
rs2032583 (c.2685+49T>C)	AFR: 0.203 EAS: 0.069 SAS: 0.180 EUR: 0.134	Variant significantly associated with SSRI adverse drug effects including 2981 participants with mental health issues [10]. Supported by <i>in vivo</i> (mice) data [6]. No significant effect on 888 patients with depressive disorders [7]

Table 7.4. Relevant non-coding SNPs in *ABCB1*.

SNPs	Frequency	<i>In vitro and/or in vivo effect</i>
rs3743527 (c.*543C>T)	AFR: 0.147 EAS: 0.439 SAS: 0.451 EUR: 0.211	Retrospective study on 235 pediatric patients associated the variant carriers (TT) with ↓ left ventricular fractional shortening after chemotherapy [11]
rs4148382 (g.16144637G>A)	AFR: 0.020 EAS: 0.062 SAS: 0.040 EUR: 0.097	Associated with a ↑ expiratory force volume in 1 second (FEV ₁) level [12] but another study associated the variant with ↓ FEV ₁ and ↑ number of sputum cells [13] in patients with chronic obstructive pulmonary disease (COPD).
rs212093 (g.16143897A>G)	AFR: 0.569 EAS: 0.699 SAS: 0.571 EUR: 0.435	Associated with ↓ FEV ₁ [12], however later associated with ↑ FEV ₁ and less inflammatory cells in patients with COPD [13].
rs4781699 (c.48+5977T>G)	AFR: 0.557 EAS: 0.708 SAS: 0.752 EUR: 0.714	Variant associated with accelerated FEV ₁ decline [12] ↓ airway wall inflammation [13]

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rs504348 (n.123+57C>A)	AFR: 0.000 EAS: 0.000 SAS: 0.000 EUR: 0.00	Variant associated with accelerated FEV ₁ decline [12] ↓ airway wall inflammation [13]	
rs4148350 (c.1988+219G>T)	AFR: 0.108 EAS: 0.050 SAS: 0.022 EUR: 0.058	Associated with febrile neutropenia in a retrospective study on 994 patients [14]	
rs246221 (2) (c.825T>C, p.275Val=)	AFR: 0.649 EAS: 0.416 SAS: 0.205 EUR: 0.301	See Chapter I – Table 1.10	Combination of rs246221TC/TT and rs3743527TT associated with ↓ left ventricular fractional shortening after chemotherapy [11]
rs35621 (c.1913-1575C>T)	AFR: 0.106 EAS: 0.218 SAS: 0.110 EUR: 0.111	Significantly associated with ↑ FEV ₁ decline [12]	

Table 7.5. Relevant non-coding SNPs in *ABCC1*.

SNPs	Frequency	<i>In vitro and/or in vivo effect</i>	
rs12762549 (g.99861014C>G)	AFR: 0.423 EAS: 0.572 SAS: 0.537 EUR: 0.479	Significantly associated with docetaxel-induced leukopenia/neutropenia on a cohort of 84 patients [2] Associated with difference in plasma levels of isoflavones metabolites [15]	
rs717620 (c.-24C>T in 3' UTR)	AFR: 0.058 EAS: 0.212 SAS: 0.113 EUR: 0.200	Associated with risk of low-response to HDL-C elevation in simvastatin treatment [16] Not associated with change in <i>ABCC2</i> placental expression [17] Associated with significant ↓ of total cholesterol and LDL-C in 302 patients treated with statins [18]	H12 haplotype (– 24T/1249G/3972T) is at ↑ risk than H2 haplotype (– 24C/1249A/3972C) to switch statin therapy [19]
rs2273697 (1) (c.1249G>A, p.Val417Ile)	AFR: 0.183 EAS: 0.088 SAS: 0.278 EUR: 0.197	See Chapter I – Table 1.11	

rs3740066 (3) (c.3972C>T, p.Ile1324=)	AFR: 0.259 EAS: 0.230 SAS: 0.325 EUR: 0.370	See Chapter I – Table 1.11	
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Table 7.6. Relevant non-coding SNPs in *ABCC2*.

SNPs	Frequency	<i>In vitro and/or in vivo effect</i>
rs3742106 (c.*38T>G in 3' UTR)	AFR: 0.322 EAS: 0.511 SAS: 0.494 EUR: 0.388	↓ plasma concentration of ATV and ATV metabolites due to ↓ efflux activity [20] Associated with ↑ B2M excretion in HIV patients [21]
rs1059751 (c.*879T>C in 3' UTR)	AFR: 0.288 EAS: 0.494 SAS: 0.479 EUR: 0.464	Homo- and heterozygous carriers associated with ↑ B2M excretion in HIV patients [21, 22] Variant predispose to renal tubular dysfunction with tenofovir disoproxil fumarate in HIV patients [23]

Table 7.7. Relevant non-coding SNPs in *ABCC4*.

SNPs	Frequency	<i>In vitro and/or in vivo effect</i>
rs2622621 (c.1195-2502G>C)	AFR: 0.159 EAS: 0.579 SAS: 0.380 EUR: 0.356	Heterozygous had ↓ risk of colorectal cancer [24] ↑ risk of gout including 143 gout patients [25]
rs1481012 (c.841+179T>C)	AFR: 0.032 EAS: 0.303 SAS: 0.095 EUR: 0.108	Heterozygous had ↓ risk of colorectal cancer [24] Associated with the development of gout in association with rs2728121 in <i>PKD2</i> [26]
rs2199936 (c.532-2387T>C)	AFR: 0.873 EAS: 0.690 SAS: 0.904 EUR: 0.890	Associated with LDL-C reduction with rosuvastatin [27] Association with incident gout [28]
rs3114018 (c.-19-3415T>G)	AFR: 0.343 EAS: 0.641 SAS: 0.556 EUR: 0.535	↑ risk of gout [25, 29] Associated with bleeding complications in patients treated with apixaban and rivaroxaban [30] Associated with ↑ risk of severe oxaliplatin-induced peripheral neuropathy [31]
rs3114020 (c.-19-22500A>G)	AFR: 0.244 EAS: 0.663 SAS: 0.458	↑ risk of gout [25] Significantly associated with lamotrigine concentrations [32]

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	EUR: 0.459		
rs7699188 (-15994C>T)	AFR: 0.448 EAS: 0.078 SAS: 0.198 EUR: 0.161	↑ BCRP expression in tissues and ↑ imatinib clearance [33] ↑ risk of severe cytotoxicity [34]	
-15622C>T	Na	↓ expression in tissues [33] Associated with ↑ erlotinib AUC and Cmax on 80 cancerous patients [35] Homo- and/or heterozygous associated with ↑ gefitinib toxicity in non-small-cell lung cancer (NSCLC) patients [36]	↑ risk of sunitinib-induced toxicity on 219 patients [37] Associated with ↑ gefitinib toxicity in NSCLC patients [36]
rs2622604 (c.-20+614A>G)	AFR: 0.874 EAS: 0.812 SAS: 0.847 EUR: 0.751	Associated with ↑ erlotinib AUC and Cmax on 80 cancerous patients [35]	

Table 7.8. Relevant non-coding SNPs in *ABCG2*.

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