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(Direktor Univ.- Prof. Dr. Werner Siegmund)
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**The role of uptake and efflux transporters in the pharmacokinetics of β 1-
receptor blocker talinolol**

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Tarek Roustom
geboren am 12.01.1989
in Hama, Syrien

Dekan: Prof. Dr. rer. nat. Max P. Bauer

1. Gutachter: Prof. Dr. med. Werner Siegmund

2. Gutachter: Prof. Dr. rer. nat. Mladen Tzvetkov

Ort, Raum: Greifswald, Institut für Pharmakologie

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1. List of abbreviations:

ABC	ATP binding cassette
AMG	Arzneimittelgesetz (=German Medicines Act)
ASBT	apical sodium-dependent bile acid transporter
AUC	area under the serum concentration-time curve
BCRP	breast cancer resistance protein
Caco-2	human epithelial colorectal adenocarcinoma cells
C_DAT	Center of Drug Absorption and Transport
C _{max}	maximum serum concentration
CL _R	renal clearance
C _{int.}	intrinsic clearance
CYP	cytochrome P450
e.g.	for example
EMA	European Medicines Agency
F	absolute bioavailability
FDA	US Food and Drug Administration
h	hour
HEK cells	human embryonic kidney cells
K _m	Michaelis-Menten constant
MDCK cells	Madin-Darby Canine Kidney Epithelial Cells
MRP	multidrug resistance related protein
NTCP	Na ⁺ -taurocholate co-transporting polypeptide
OATP	organic anion transport polypeptides
OCT	organic cation transporter
OCTN	organic cation/carnitine transporter
PEPT	peptide transporter
P-gp	P-glycoprotein
SNP	single-nucleotide polymorphism
SLC	solute carrier
T _{1/2}	elimination half life
T _{max}	time of maximal concentration
V _d	volume of distribution
V _{max}	maximal uptake rate

2. Introduction:

In the practice of current pharmacotherapy and with more drugs being approved every year, drug related adverse events are believed to be responsible for more than five percent of the hospitalizations leading in Germany alone to about 50,000 deaths annually.^{1,2} Some major causes of severe adverse drug reactions are pharmacogenomic factors, drug-drug and food-drug interactions or organ diseases. To optimize drug treatment by individualization and to minimize drug safety, there is an urgent need to understand the mechanisms behind the variability in pharmacokinetics of drugs.

The drug biotransformation has been considered for a long time to be the major confounder for pharmacokinetic and drug interactions.^{3,4} After many years of intensive research, the metabolism of drugs is well characterized and a plenty of *in-vitro* and *in-vivo* probe drugs are validated to measure the functions of drug metabolizing enzymes and to predict variability as caused by the major confounders, e.g. midazolam and caffeine as probe drugs for CYP3A4 and CYP1A2, respectively.^{5,6} However, biotransformation is only one variable for drug efficacy and safety. Therefore, only a part of the overall variability can be explained by particularities in metabolic drug disposition. This leaves the door open for other biological confounders to be investigated.

The discovery of the multidrug resistance P-glycoprotein in 1986, the first member of the ABC (ATP binding cassette) transporter family, has initiated a new field in pharmacokinetics. In the recent decades, this field has been rapidly expanding with new families and subfamilies of transport proteins being identified and characterized in localization, expression and function. Regarding their function, transporters can be classified as uptake or efflux transporters which mediate the membrane transport of endogenous compounds, xenobiotics and drugs. Uptake transporters mediate the cellular influx of their substrates in parenchymal cells (e.g. enterocytes, hepatocytes, tubular cells in the kidneys). Efflux transporters in the intestine, liver, kidney, brain, testes, and placenta serve as functional barriers against the entry of many xenobiotics into the body and many organs (e.g.

intestinal absorption barrier, blood-brain barrier, placenta barrier) and enable elimination via kidneys and intestine. Therefore, both uptake and efflux transporters are major variables in absorption, distribution and excretion of drugs.⁷

The clinical relevance of a transporter is highly determined by its function, substrate specificity, expression level in the different tissues and the presence/absence of other transporters with overlapping substrate preferences. So far, numerous transporter proteins are known for having a relevant impact on drug pharmacokinetics. These are particularly the organic anion transporting polypeptides (OATPs), the organic anion transporters (OATs), the organic cation transporters (OCTs) and the peptide transporters (PEPTs) as members of the solute carrier superfamily (SLC), as well as the members of ABC-superfamily P-glycoprotein (P-gp), the breast cancer resistance protein (BCRP) and multidrug resistance related protein (MRPs).⁷

With the data accumulated so far, the clinical importance of drug transporters in absorption, distribution and elimination of drugs can no longer be ignored, so that it is becoming mandatory for new chemical entities to be tested for transporter affinity in very early stages of the preclinical and clinical development to evaluate their interactive potential *in-vitro* and *in-vivo* according to the recommendations of the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA).

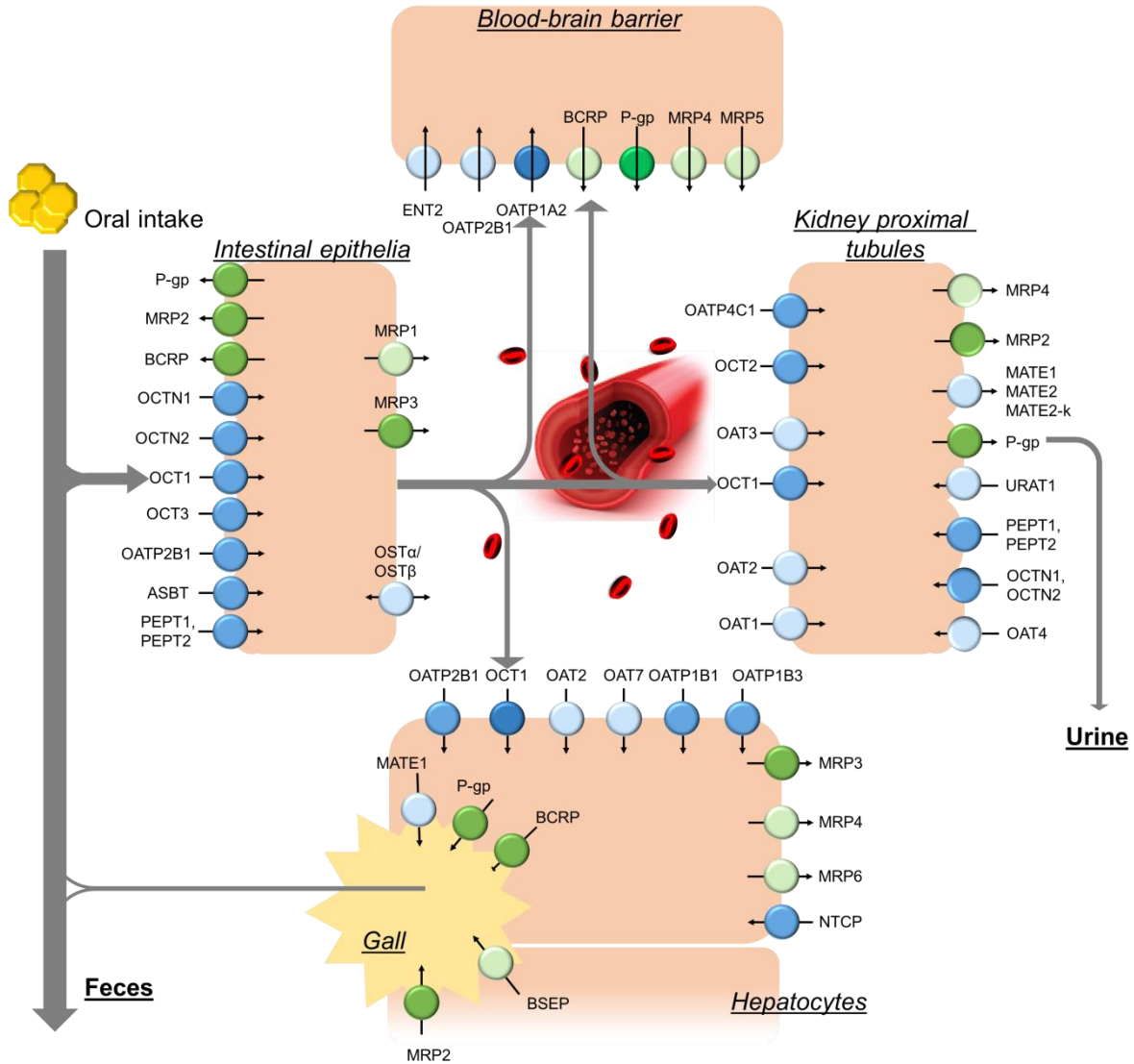


Figure 1: Transporters in plasma membrane domains of intestinal epithelia, hepatocytes, kidney proximal tubules and brain capillary endothelial cells (modified according to Hillgren et al. 2013).⁸

Additionally, the huge methodological progress in pharmacogenomics, particularly in genotyping and SNPs detection methods enabled the challenging new conception of the personalized medicine, in which transporter proteins serve as an important determinant of the inter-individual variability in drug pharmacokinetics. These facts promote the need for characterizing the relevant transporters and evaluation of appropriate *in-vitro* and *in-vivo* probe drugs that can be reliably used to predict variability in transporter function as caused by genetic and non-genetic factors mentioned above.

For the necessary *in-vitro* testing of transporter functions, numerous chemical substances have been used successfully as probe drugs (substrates or inhibitors) for particular transporters, e.g., the use of estradiol-17 β -glucuronide (E-17 β -Gln) or sulfobromophthalein (BSP) as a substrate of OATP1B1/1B3, 1-methyl-4-phenylpyridinium (MPP+) as a substrate of OCT1/3 and many others.^{9,10} Nevertheless, and despite the importance of *in-vitro* testing in order to gain a basic understanding of the function and substrate spectrum of the different transporters, this *in-vitro* testing alone is not suitable to fully predict pharmacokinetics of drugs *in-vivo*. This is due to the fact that pharmacokinetics includes a complex interplay of many factors, in which transport proteins, drug metabolizing enzymes in addition to the changing physical and chemical environment are involved. Therefore, it is essential to identify *in-vivo* probe drugs that can be safely administered in man to provide us with reliable data about the specific protein transporters.

3. Background and rationale:

An *in-vivo* probe drug for a protein transporter should be a selective substrate of the studied transporter, safe and well tolerated by healthy subjects and should not be metabolized or undergo only minor metabolism. Additionally, it is very important to know the impact of other transporters involved in absorption, distribution and elimination of the respective probe drug.

One well-known example is the cardiac glycoside digoxin, which has been used in numerous clinical studies as an *in-vivo* probe drug for intestinal P-gp as it meets many of the above mentioned criteria. Unfortunately, the use of digoxin for this purpose faces several limitations:¹¹

- Digoxin undergoes a biotransformation so that about 15% of an oral dose is metabolized by sugar cleavage and glucuronidation.
- Other transporters like OATP1B3 and OATP4C1 seem to be involved in the pharmacokinetics of digoxin and, therefore, need to be considered in any clinical study.
- Due to its long terminal half-life, pharmacokinetic studies with digoxin require sampling (plasma, urine, feces) for at least 7 days. Therefore, controlled studies with digoxin are of long duration and of high risk for dropping out and the subsequent effects.
- Analytical problems are likely in pharmacokinetic studies with digoxin because of the low average C_{max} -values of 1-5 ng/ml after single oral doses of 0.25-1.0 mg.

An alternative candidate to digoxin is the selective β_1 -receptor blocker talinolol, which was launched on the German market for treatment of arterial hypertension and coronary heart disease. Talinolol is given in a standard dose of 100 mg per os which is considered to be safe and well tolerated in studies applying single or repeated doses in healthy subjects.¹²⁻¹⁵ In these studies, no adverse events were reported as probably or likely related to the study medication. Even single doses of

400 mg were well tolerated.¹⁶ Therefore, talinolol can be safely used as a study medication in healthy subjects.

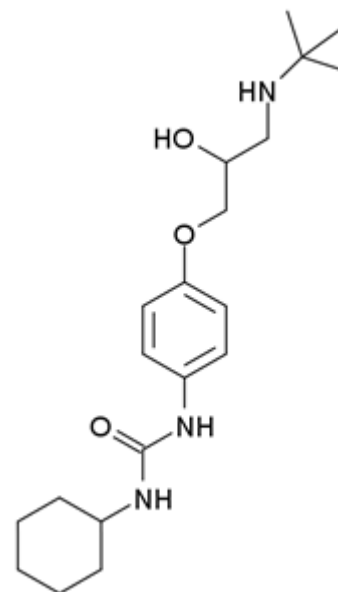
Talinolol is a drug with high water solubility and low permeability (pH 7.4: 1.24 g/l; pH 7.0: 4.5 g/l; 37C).^{17,18} It is erratically and incompletely absorbed from the gastrointestinal tract with an absolute bioavailability about 55 - 70 % and is widely distributed (Vd = 3 - 6 l/kg). 50 - 70 % are bound to plasma proteins.^{12,13,15,19-21} The drug is nearly not metabolized with less than 1 % of the dose being transformed to 4-trans and 3-cis-hydroxy-talinolol.

^{12,13,15,19}

When given intravenously, 3.7 - 25 % (median: 9.3 %) of talinolol is excreted into the bile resulting in

biliary concentrations up to 90-fold above the serum levels.²² After intravenous administration, about 43 % of the dose is excreted with the urine, and about 22 % is recovered in the feces, while an oral administration in a dose of 100 mg results in about 30 % excreted with urine and another 30 % appearing in feces.²³ Renal clearance is about 150–190 ml/min which is higher than the filtration rate in healthy subjects (~120 ml/min) which may be explained by the contribution of an active tubular secretion to the renal elimination.^{12,13,15,19,23}

The utility of talinolol as an *in-vivo* probe drug for the P-gp efflux transporter is supported by many experiments and clinical observations. Transport experiments using Caco-2 cell monolayers suggested that talinolol is a P-gp substrate, as its basolateral to apical transport was considerably higher than in the opposite direction. Moreover, this difference in transport rate was significantly decreased in the presence of strong inhibitors of P-gp like verapamil, LY335984, constituents of grapefruit juice or surfactants.²⁴⁻²⁹ Similar results were observed by assessing the serosal-mucosal permeability of talinolol in everted sacs of rat ileum.³⁰ Further confirmation came from experiments with the P-gp inducer rifampicin which decreased the intestinal permeability of talinolol in the duodenum, jejunum and



colon of rats (in situ perfusion).³¹ Furthermore, bioavailability of oral talinolol in rats increased significantly after co-medication of grapefruit juice or some of its constituents like the P-gp inhibitors naringin or bergamottin.^{25,32,33} In *Abcb1a/1b* (-/-) knock-out mice, the apparent permeability for talinolol in perfused intestinal tissue increased sevenfold.³⁴ The plasma levels of talinolol in knock-out mice after oral administration exceeded nearly threefold their levels in wildtype mice.³⁵

The first *in-vivo* evidence came from a clinical study in healthy subjects by Gramatté et al. who proved the active intestinal secretion of talinolol in man using an intestinal steady-state perfusion method.^{36,37} Further clinical evidence for the applicability of talinolol as a probe drug for intestinal P-gp came from interaction studies with the P-gp inducers rifampicin and Saint John's wort (SJW) which reduced talinolol bioavailability by 21% and 25%, respectively.^{19,38} The impact of P-gp inhibition on pharmacokinetics of oral talinolol has been proven by co-administration of talinolol and erythromycin.^{39,40}

As a characteristic feature of P-gp substrates, regio-selective absorption was also suggested for talinolol. This was confirmed by the data on systemic availability of talinolol simultaneously measured by triple lumen tubes perfusion.^{36,37} AUC and C_{max} decreased with increasing distance of the perfusion port from the teeth by up to 85 %. The results of both perfusion studies reflected the findings on regio-selective expression of intestinal P-gp published several years later.^{41,42} This behavior was confirmed in a clinical study with talinolol prepared in immediate release hard gelatin capsules and enteric-coated sustained release hard gelatin capsules.⁴³

Along with the convincing evidence on intestinal active secretion of talinolol in man, some unexpected pharmacokinetic observations appeared to challenge the conception that talinolol is a selective probe-drug for intestinal P-gp.³⁵⁻³⁷ In a drug interaction study in healthy subjects with concomitant oral administration of talinolol and R-verapamil, an increase of talinolol bioavailability was expected as R-verapamil is an inhibitor of P-gp.^{44,45} Instead, a significantly reduced AUC was surprisingly observed with no change in half-life or renal clearance. Again, similar results were seen in experiments with *Abcb1a/b* (-/-) knock-out mice, where the

plasma levels of talinolol were, as expected, higher in deficient than in wild-type animals.³⁵ However, co-administration of verapamil resulted in significantly lower talinolol concentrations in both groups suggesting an involvement of intestinal uptake transporters of talinolol, whose inhibition may have overshadowed the effects of verapamil on P-gp. Further evidence for the existence of an unknown uptake transporter for talinolol came from a clinical study with talinolol administered with single and repeated ingestion of grapefruit juice. Under both conditions AUC and C_{max} of talinolol were lowered by more than 50 % whereas half-life and renal clearance remained unchanged. These findings were contrary to the study hypothesis to which the authors had expected higher bioavailability of talinolol because grapefruit juice and its constituents inhibit the unidirectional talinolol transport in Caco-2 cells and increase talinolol absorption in rats.^{25,32,33} Therefore, and before talinolol can be efficiently used as an *in-vivo* probe drug, its influx and efflux mechanisms through the different body barriers need to be well understood.

The objectives of this paper were:

- To assess the *in-vitro* affinity of talinolol to drug transporting proteins and some of their genetic variants known to be of pharmacokinetic relevance. The studied transporters were: organic cation transporters (OCT1-3), organic anion transport polypeptides (OATP 1B1, 1B3, 2B1 and 1A2), peptide transporter 1 (PEPT1), organic cation/carnitine transporter 2 (OCTN2), apical sodium-dependent bile acid transporter (ASBT), Na⁺-taurocholate co-transporting polypeptide (NTCP) and multidrug resistance related proteins (MRP 1-3), in addition to the genetic polymorphisms of OATP1A2 (*2 and *3) and of OATP2B1 (V201M, R312Q and S486F).
- To retrospectively analyze the studies conducted with talinolol in the Department of Clinical Pharmacology in order to evaluate the impact of clinically relevant transporter genetic polymorphisms on the pharmacokinetics of talinolol in healthy subjects. The genetic polymorphisms included in this evaluation are listed in Table 3, and were chosen for being known to affect the expression or the function of the respective transporters.

4. Materials and methods:

4. 1. *In-vitro* Studies:

4.1.1. Chemicals:

Taurocholate (TA), estrone-3-sulfate (E1S), N-methyl-4-phenylpyridinium (MPP+), estradiol-17 β -glucuronide E-17 β -Gln, Glycyl-sarcosine (Gly-Sar), rifampicin (Rifa), bromosulfophthalein (BSP), rhodamine-123 (Rh123), verapamil and MK-571 were obtained from Sigma-Aldrich (Taufkirchen, Germany). Talinolol was purchased from the former Arzneimittelwerk Dreseden (AWD, Dresden, Germany).

[³H]-BSP (14 Ci/mmol; 1 μ Ci/ μ l), [³H]-E-17 β -Gln (41,8 Ci/mmol), [³H]-Gly-Sar (2 Ci/mmol; 1 μ Ci/ μ l) and [³H]-talinolol (20 Ci/mmol) were obtained from Hartmann Analytic (Braunschweig, Germany), [³H]-TA (4.6 Ci/mmol; 1 μ Ci/ μ l) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, USA), [³H]-MPP (85 Ci/mmol; 1 μ Ci/ μ l) and [³H]-E1S (50 Ci/mmol; 1 μ Ci/ μ l) from Biotrend (Cologne, Germany). PSC-833 was kindly provided by Novartis (Basel, Switzerland).

4.1.2. Cell culture:

Human embryonic kidney 293 (HEK293) cells and Madin-Darby canine kidney (MDCK2) cells were purchased from the European Collection of Cell Cultures (Salisbury, United Kingdom). HEK293 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 2 mM nonessential amino acids, 100 units/ml penicillin and 100 μ g/ml streptomycin. MDCK2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin (PAA, Coelbe, Germany) at 37 °C, 95% humidity, and 5% carbon dioxide.

HEK293 cells were stably transfected with human OATP1A2 *1, *2 and *3, OATP1B1, OATP1B3, OATP2B1 (and its genetic variants p.V201M, p.R312Q and p.S486F), NTCP, ASBT, OCT 1-3 and the respective vector control, while MDCK2 cells were stably transfected with PEPT1, OCTN2 and the respective vector control. The transfected cells were established as previously described⁴⁶⁻⁵⁰.

4.1.3. Affinity assays with uptake transporters:

For uptake and competition assays, cells were seeded in 24-well plates and incubated in full growth medium at an initial density of 100000 cells/well for 3 days. Before each experiment, cells were washed once with 37 °C incubation buffer (142 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l K₂HPO₄, 1.2 mmol/l MgSO₄, 1. mmol/l CaCl₂, 5 mmol/l glucose, and 12.5 mmol/l HEPES; pH 7.3). After the respective uptake or competition experiment, cells were washed three times with ice-cold PBS and lysed with 800 µl of room-temperature 0.5% Triton X-100 (Merck, Darmstadt, Germany) and 0.5% sodium deoxycholate (Sigma-Aldrich, Steinheim, Germany). 100 µl of cell suspension were mixed with 1 ml of scintillation cocktail (Rotiszint ecoplus; Roth, Karlsruhe, Germany) and measured using a scintillation beta counter (type 1409, LKB-Wallac, Turku, Finland). Total cell protein content was determined to quantify cell density after the experiments using the BCA assay according to the manufacturer's instructions (Pierce, Rockford, USA).

In competition assays, the reference substrates [³H]-BSP, [³H]-E1S, [³H]-TA, [³H]-MPP⁺ and [³H]-Gly-Sar were dissolved in incubation buffer, and unlabeled BSP, E1S, TA, MPP⁺, and Gly-Sar were added to reach the respective final concentration of 0.05 µmol/l in OATP1B1, 1 µmol/l in OATP1A2*1, *2, and *3, OATP1B3, and OATP2B1 (and its genetic variants), and 10 µmol/l in OCT1-3, NTCP, PEPT1, OCTN2 and ASBT transfected cells. Inhibitory effects of talinolol were investigated in all cell lines using the above mentioned reference substrates and talinolol at concentrations up to 1000 µmol/l.

The uptake activities of the transporters were measured using the labeled reference substrates dissolved with their unlabeled equivalents for predefined final concentrations. The cellular uptake of all reference substrates was in the linear range during an incubation time of 10 sec for OCT1-2, 60 sec for OCT3, PEPT1 and 5 min for OATPs, ASBT, NTCP and OCTN2 transfected cells.

Uptake of talinolol by OATPs, NTCP, ASBT, OCTs, PEPT1 and OCTN2 was first measured in time-dependent uptake studies for up to 30 min with [³H]-talino-
lolol dissolved with unlabeled talinolol to reach a final concentration of 100 µmol/l. The

concentration dependent uptake was then measured with a final talinolol concentration up to 1000 $\mu\text{mol/l}$. During the respective incubation times, the cellular substrate influx was in its linear phase still before reaching equilibrium.

4.1.4. Affinity assays with efflux transporters:

Membrane transport of talinolol mediated by MRP1-3 and P-gp was investigated using inside-out vesicles. For all experiments, 30 μg of total vesicle protein were used. The preparation of inside-out vesicles from stably transfected MDCK2-MRP2 and HEK293-Pgp was performed as described previously.^{51,52} MRP1 and 3 vesicles were purchased from BD Biosciences (Darmstadt, Germany). Functionality of P-gp lipovesicles was ensured by an Rh123 accumulation assay in the presence or absence of ATP. Fluorescence of Rh123 was measured using a microplate reader (Synergy HT, Biotek, Bad Friedrichshall, Germany). Functionality of MRP1-3 was ensured as described previously.^{50,52}

ATP dependent transport of [³H]-talinolol and unlabeled talinolol (0-1000 $\mu\text{mol/l}$) into the vesicles was measured by rapid filtration using nitrocellulose filters (0.22 μm pore size; Millipore, Billerica, USA). In control experiments, ATP was replaced by AMP. In competition assays, P-gp or MRP vesicles were incubated with radiolabeled talinolol and 10 $\mu\text{mol/l}$ of the known P-gp inhibitor PSC-833⁵³ or the MRP-inhibitor MK-571, respectively. The calculation and correction of the different parameters was made as described below.

4.1.5. Correction of talinolol transport:

In order to have comparable affinity and capacity values for the different transporters which have shown a positive concentration dependent transport of talinolol, the specific protein content of each of these transporters was measured by a validated LC-MS/MS method as described previously.⁵⁴

The transport rates were then attributed to the transporter proteomics measured in the cellular membrane and presented as pmol/mg total cell protein (Table 1). The

corrected transport data were then used to calculate K_m , V_{max} and $C_{int.}$ for each transporter.

Table 1: Mean cellular protein abundance as measured by LC-MS/MS.

Transporter protein	Specific protein abundance (pmol/mg total cell protein)
P-gp	20.0
MRP3	0.42
OATP1B1	2.38
OATP1B3	0.14
OATP2B1	8.97
OATP2B1_RQ	11.3
OATP2B1_SF	5.50
OATP2B1_VM	2.18
OATP1A2	1.90
OATP1A2 *2	5.17
OATP1A2 *3	2.86
NTCP	0.29

4.1.6. Biometrics

The program Prism 5.01 (GraphPad Software, San Diego, USA) was used to evaluate the experiments. Michaelis-Menten constant (K_m) and the maximal uptake rate (V_{max}) values were calculated using the Michaelis-Menten nonlinear regression model and presented as mean value and standard deviation (mean \pm SD). The intrinsic clearance ($C_{int.}$) was calculated as quotient from V_{max} and K_m .

4.2. Retrospective pharmacogenomic analysis:

4.2.1. Data collection:

The pharmacogenomic evaluation was carried out for 39 healthy subjects who participated in former pharmacokinetic studies with talinolol and for whom, DNA or blood samples were available. The pharmacokinetic data of four studies (Table 2) were included in our analysis responding to the following criteria:

1. The studies were conducted in the Department of Clinical Pharmacology, University Medicine Greifswald between 2000 and 2015 in subjects who had given written informed consent for a pharmacogenomic analysis before inclusion into the respective studies.
2. The inclusion and exclusion criteria for healthy subjects were similar in all clinical studies with talinlolol with no relevant differences.
3. All subjects received talinolol as a single dose, without any co-medication that might have an influence on talinolol pharmacokinetics.

The pharmacokinetic data were taken from the database of the Department of Clinical Pharmacology, University Medicine Greifswald. The use of the data in completely anonymized manner was in agreement with the written informed consents for inclusion in clinical studies at the Department of Clinical Pharmacology and of the former clinical studies with talinolol which were approved by the Ethics Committee of the University Medicine Greifswald.

Table 2: Detailed information about the studies involved in the retrospective analysis

Study code	Protocol title	Study design	Sample size
Talinolol-Digoxin- 0298	Oral bioavailability of digoxin is enhanced by talinolol: Evidence for involvement of intestinal P-glycoprotein ¹⁹	controlled, open, randomized, single-dose, five period and change-over	5
Cordan- 3010	Simvastatin does not influence the intestinal P-glycoprotein and MPR2, and the disposition of talinolol after chronic medication in healthy subjects genotyped for the ABCB1, ABCC2 and SLCO1B1 polymorphisms ²³	controlled, randomized, open, single and multiple dose, five-period and change-over	13
Talinolol-Lymphe	The talinolol double-peak phenomenon is likely caused by presystemic processing after uptake from gut lumen. ⁴³	controlled, randomized, open, single-dose, four period and change-over	4
Talino-8014	Variability of intestinal expression of P-glycoprotein in healthy volunteers as described by absorption of talinolol from four bioequivalent tablets. ¹⁴	single dose, controlled, randomized, open, four period change-over trial in healthy volunteers	17

4.2.2. Genotyping:

All individuals included in the retrospective analysis were genotyped for a number of frequently occurring polymorphisms (Table 3). The genotyping was performed for all polymorphisms, with one exception, using predeveloped TaqMan SNP detection assays from Applied Biosystems (Darmstadt, Germany). Briefly, reactions were carried out in a volume of 10 μ l containing 5 μ l Genotyping Master Mix (Applied Biosystems, Darmstadt, Germany) 1 μ l genomic DNA and 0.5 μ l of the Primer Probe-Mix. Fluorescence was assessed using the Fast Real-Time PCR system 7900 HT (Applied Biosystems, Darmstadt, Germany) and the Sequence

Detection Software SDS 2.3 (Applied Biosystems, Darmstadt, Germany). The rs4149056 (c.SLCO1B1 521T > C) polymorphism was detected in a genome-wide SNP scan using the Affymetrix Human SNP Array 6.0 (Affymetrix Inc., Santa Clara, California, USA) (SNP_A-860113). Data on SNP_A-860113 were extracted and used for statistical analysis.

The previous clinical studies were performed according to the ICH-GCP-guidance for clinical studies and to the German Medicines Act §§ 40-42. All subjects had provided written informed consent for genotyping of all relevant genetic polymorphisms in drug metabolism and transport before inclusion into the respective clinical study and use of the data in completely anonymized form. All procedures and clinical studies had been approved by the Ethics Committee of the University Medicine Greifswald.

4.2.3. Pharmacokinetic and biometric evaluation:

The pharmacogenomic analysis was conducted using the following pharmacokinetic parameters: area under the serum concentration-time curve (AUC), bioavailability (F), maximum serum concentration (C_{max}), time of maximum concentration (T_{max}), elimination half-life ($T_{1/2}$) and the renal clearance (CL_R).

For all samples, arithmetic means and standard deviations (mean \pm SD) are given. Sample differences were evaluated using the Kruskal-Wallis with post hoc tests and Mann-Whitney-U test, assuming statistical significance at a level of $p < 0.05$, and the SPSS software package (version 22; IBM, NY, USA).

Table 3: The single nucleotide polymorphisms (SNPs) and assays for genotyping of healthy subjects included in the retrospective pharmacogenomic analysis.

Gene	Protein	SNP ID	SNP Type	Codon Change	Amino Acid Change
<i>ABCB1</i>	P-gp	rs1128503	silent	GGC,GGT	G412G
<i>ABCB1</i>	P-gp	rs2032582	missense	CCT,TCT	P893S
<i>ABCB1</i>	P-gp	rs1045642	silent	-	-
<i>ABCB2</i>	MRP2	rs717620	UTR 5	-	-
<i>ABCC2</i>	MRP2	rs8187710	missense	TAC,TGC	Y1515C
<i>ABCC2</i>	MRP2	rs2273697	missense	ATT,GTT	I417V
<i>ABCC2</i>	MRP2	rs3740066	silent	ATC,ATT	I1324I
<i>ABCC3</i>	MRP3	rs4793665	intron	-	-
<i>SLC01B1</i>	OATP1B1	rs2306283	missense	AAT,GAT	N130D
<i>SLC01B1</i>	OATP1B1	rs4149056	missense	GCG,GTG	A174V
<i>SLC01B3</i>	OATP1B3	rs4149117	missense	GCT,TCT	A112S
<i>SLC22A1</i>	OCT1	rs72552763	shift delete	-	-
<i>SLC22A1</i>	OCT1	rs55918055	missense	CGC,TGC	R88C
<i>SLC22A1</i>	OCT1	rs12208357	missense	CGC,TGC	R61C
<i>SLC01A2</i>	OATP1A2	rs11568563	missense	GAA,GAC	E172D
<i>SLC01A2</i>	OATP1A2	rs10841795	missense	ACA,ATA	T13I

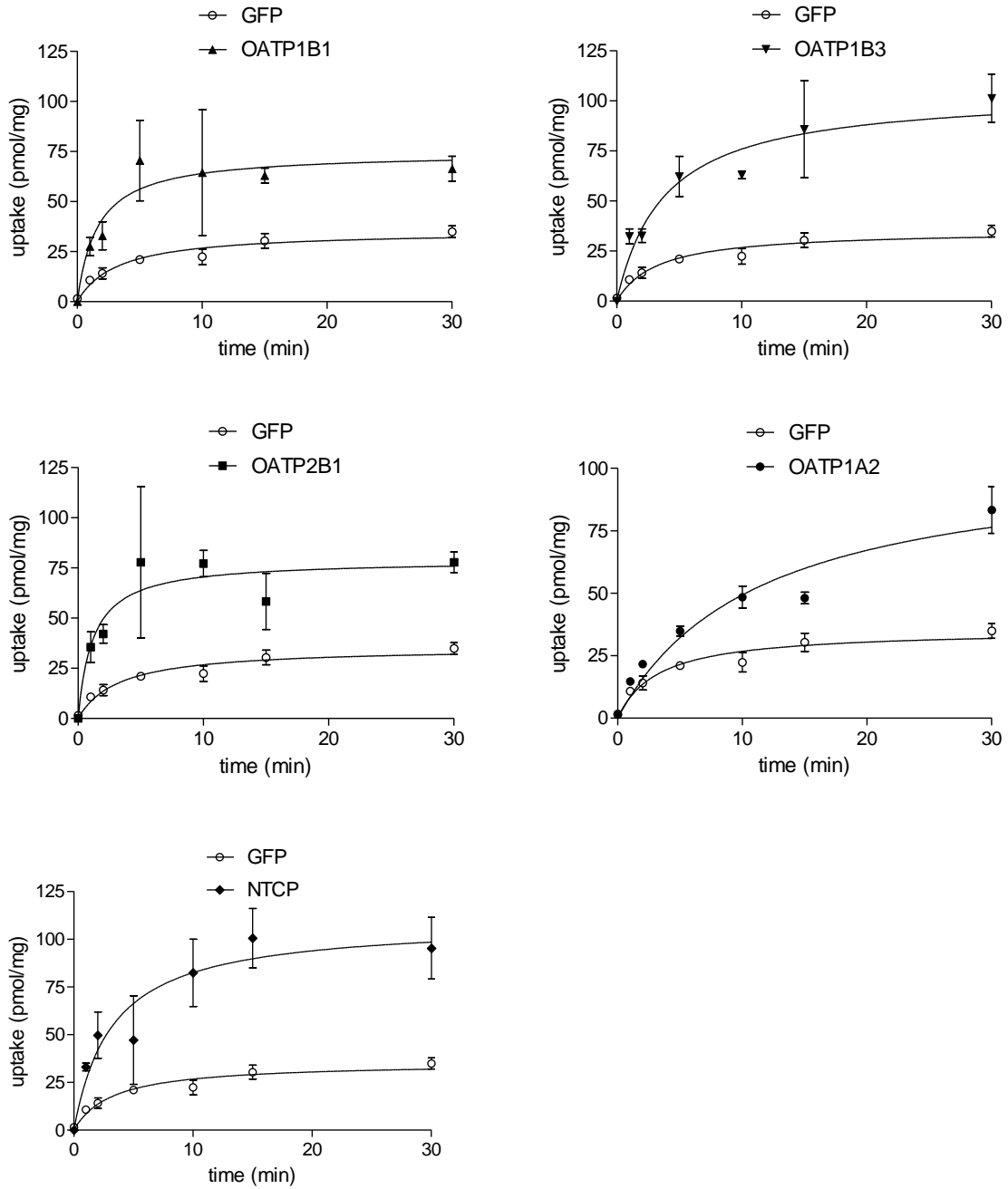
5. Results:

5.1. Affinity of talinolol to uptake transporter proteins:

OATP1B1, OATP1B3, OATP2B1, OATP1A2 and NTCP

In our *in-vitro* studies, HEK293 Cells stably transfected with human OATP1B1, OATP1B3, OATP2B1, OATP1A2 and NTCP have shown a higher time dependent uptake for talinolol comparing with similar cell lines transfected with only the empty vector (Figure 2).

The next step was to conduct concentration dependent uptake assays with these transport proteins in order to assess their affinity and capacity regarding the transport of talinolol (Figure 3, Table 4). After subtracting the uptake of cell line transfected with the empty vector (GFP), all five uptake transporters have shown a positive concentration dependent uptake for talinolol. Among these uptake transporters, it seems that OATP1B3 has the highest affinity to talinolol with K_m of $153 \pm 137 \mu\text{mol/l}$ and a relatively high capacity with V_{max} of $1135 \pm 348 \mu\text{mol/mgxmin}$. The second relevant transporter is OATP1B1 with K_m of $301 \pm 133 \mu\text{mol/l}$ and a relatively high capacity with V_{max} of $168 \pm 30.3 \mu\text{mol/mgxmin}$. OATP2B1 and OATP1A2 were shown to have both lower affinity and lower capacity than the former two transporters. OATP2B1 had K_m of $459 \pm 260 \mu\text{mol/l}$ and V_{max} of $4.32 \pm 1.33 \mu\text{mol/mgxmin}$ while OATP1A2 had K_m of $477 \pm 158 \mu\text{mol/l}$ and V_{max} of $0.61 \pm 0.1 \mu\text{mol/mgxmin}$. On the other hand, NTCP seemed to have the lowest affinity but the highest capacity for talinolol as its K_m was $2560 \pm 781 \mu\text{mol/l}$ and V_{max} of $15944 \pm 3741 \mu\text{mol/mgxmin}$. These data were used to calculate the intrinsic clearance (C_{int}) which were 0.56 ± 0.27 , 7.54 ± 7.12 , 6.23 ± 2.40 , $(10 \pm 4) \times 10^{-3}$, $(1.3 \pm 0.5) \times 10^{-3} \text{ ml}/\mu\text{gxmin}$ for OATP1B1, OATP1B3, NTCP, OATP2B1, OATP1A2, respectively.



*GFP: control vector

Figure 2: Time dependent uptake ($M \pm SD$) of talinolol in stably transfected HEK293 cells expressing OATP1B1, OATP1B3, OATP2B1, OATP1A2 and NTCP.

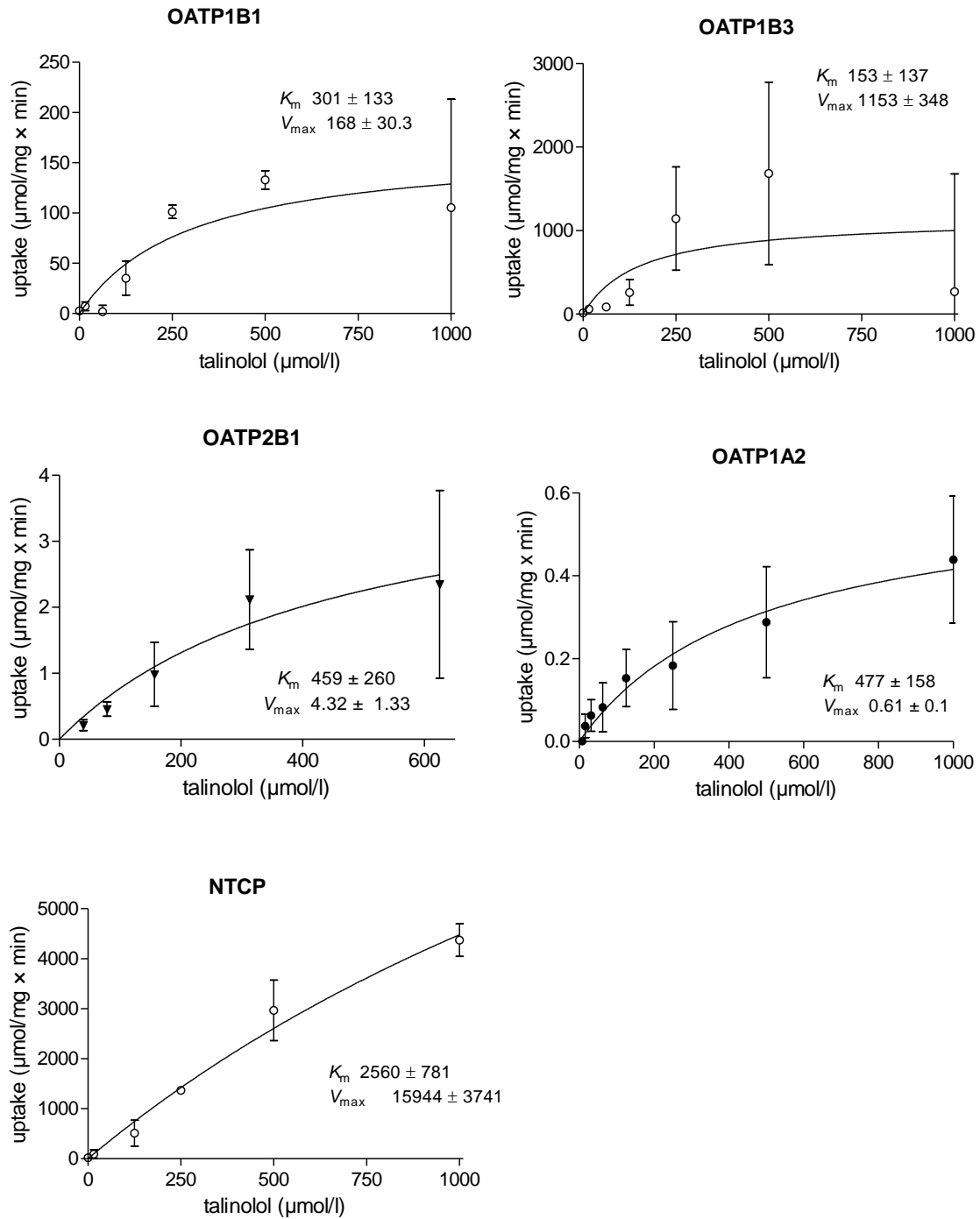


Figure 3: Concentration dependent net-uptake ($M \pm SD$) of talinolol (- control vector GFP) in stably transfected HEK293 cells expressing OATP1B1, OATP1B3 and NTCP (corrected to the specific protein content).

Furthermore, we have conducted the concentration dependent uptake assays in cells transfected with three genetic polymorphisms of OATP2B1 namely p.V201M, p.R312Q and p.S486F and with two genetic polymorphisms of OATP1A2, namely OATP1A2*2 and OATP1A2*3. Their affinity and capacity were calculated and compared with the respective wildtype in order to assess any potential influence on the transport of talinolol.

For OATP2B1, all three studied polymorphisms had lower affinity but higher capacity than the wildtype. p.V201M had K_m of $1115 \pm 642 \mu\text{mol/l}$, V_{max} of $46.7 \pm 19 \mu\text{mol/mgxmin}$ and $C_{\text{int.}}$ of $0.04 \pm 0.03 \text{ ml}/\mu\text{gxmin}$. p.R312Q had K_m of $2201 \pm 2100 \mu\text{mol/l}$, V_{max} of $15.4 \pm 12 \mu\text{mol/mgxmin}$ and $C_{\text{int.}}$ of $0.01 \pm 0.01 \text{ ml}/\mu\text{gxmin}$. p.S486F had K_m of $988 \pm 601 \mu\text{mol/l}$, V_{max} of $14 \pm 5.83 \mu\text{mol/mgxmin}$ and $C_{\text{int.}}$ of $0.01 \pm 0.01 \text{ ml}/\mu\text{gxmin}$ (Figure 4, Table 4).

In contrast, the studied polymorphisms of OATP1A2 seemed to have a higher affinity but a lower capacity than the wildtype. OATP1A2*2 had K_m of $342 \pm 268 \mu\text{mol/l}$, V_{max} of $0.08 \pm 0.03 \mu\text{mol/mgxmin}$ and $C_{\text{int.}}$ of $(0.2 \pm 0.2) \times 10^{-3} \text{ ml}/\mu\text{gxmin}$ while OATP1A2*3 had K_m of $211 \pm 219 \mu\text{mol/l}$ and V_{max} of $0.11 \pm 0.05 \mu\text{mol/mgxmin}$ and $C_{\text{int.}}$ of $(0.5 \pm 0.6) \times 10^{-3} \text{ ml}/\mu\text{gxmin}$ (Figure 5, Table 4).

Table 4: Talinolol uptake in stably transfected HEK-cells expressing OATP1B1, OATP1B3, NTCP, OATP2B1, OATP1A2 and the genetic polymorphisms of OATP2B1 (p.V201M, p.R312Q and p.S486F) and of OATP1A2 (*2 and *3).

Cell line/ Transporter	K_m ($\mu\text{mol/l}$)	V_{max} ($\mu\text{mol/mgxmin}$)	$C_{int.}$ ($\text{ml}/\mu\text{gxmin}$)
HEK293-OATP1B1	301 \pm 133	168 \pm 30.3	0.56 \pm 0.27
HEK293-OATP1B3	153 \pm 137	1153 \pm 348	7.54 \pm 7.12
HEK293-NTCP	2560 \pm 781	15944 \pm 3741	6.23 \pm 2.40
HEK293- OATP2B1 (WT)	459 \pm 260	4.32 \pm 1.33	0.01 \pm 0.004
HEK293- OATP2B1 (V201M)	1115 \pm 642	46.7 \pm 19	0.04 \pm 0.03
HEK293- OATP2B1 (R312Q)	2201 \pm 2100	15.4 \pm 12	0.01 \pm 0.01
HEK293- OATP2B1 (S486F)	988 \pm 601	14 \pm 5.83	0.01 \pm 0.01
HEK293- OATP1A2	477 \pm 158	0.61 \pm 0.1	0.001 \pm 0.0005
HEK293- OATP1A2*2	342 \pm 268	0.08 \pm 0.03	0.0002 \pm 0.0002
HEK293- OATP1A2*3	211 \pm 219	0.11 \pm 0.05	0.0005 \pm 0.0006

OCT1, OCT2, OCT3, OCTN2, PEPT1 and ASBT

Time dependent uptake assays were conducted using MDCK2 cells stably transfected by OCT1, OCT2, OCT3, OCTN2 and PEPT1 and with HEK293 cells stably transfected by ASBT. When comparing the uptake rate of these transfected cells with the control cells, one of the above mentioned transporter proteins could demonstrate a significant increase in talinolol uptake (Figure 6).

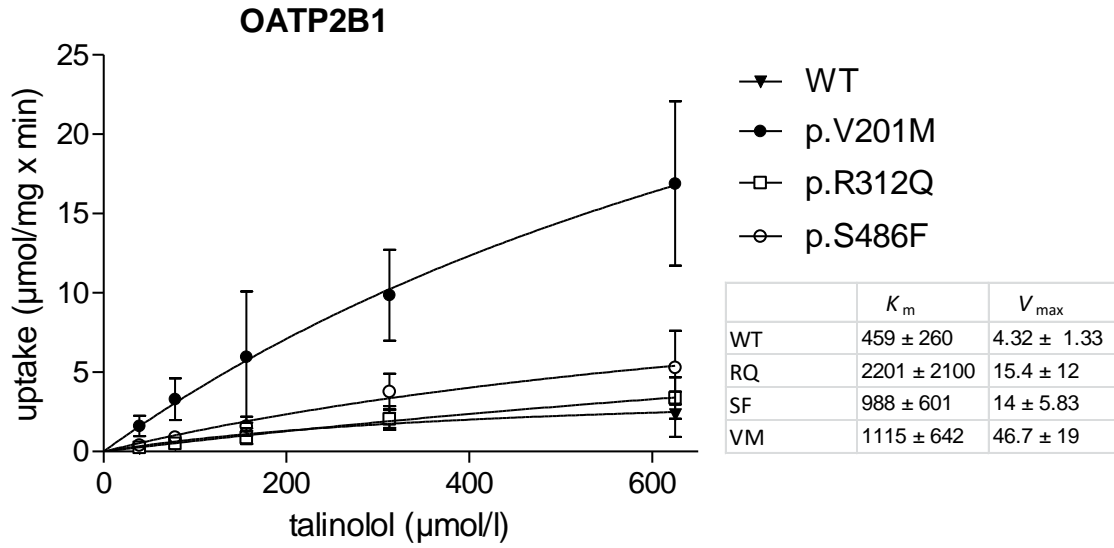


Figure 4: Concentration dependent net-uptake ($M \pm SD$) of talinolol (- control vector GFP) in stably transfected HEK293 cells expressing OATP2B1 and its genetic variants V201M, R312Q and S486F.

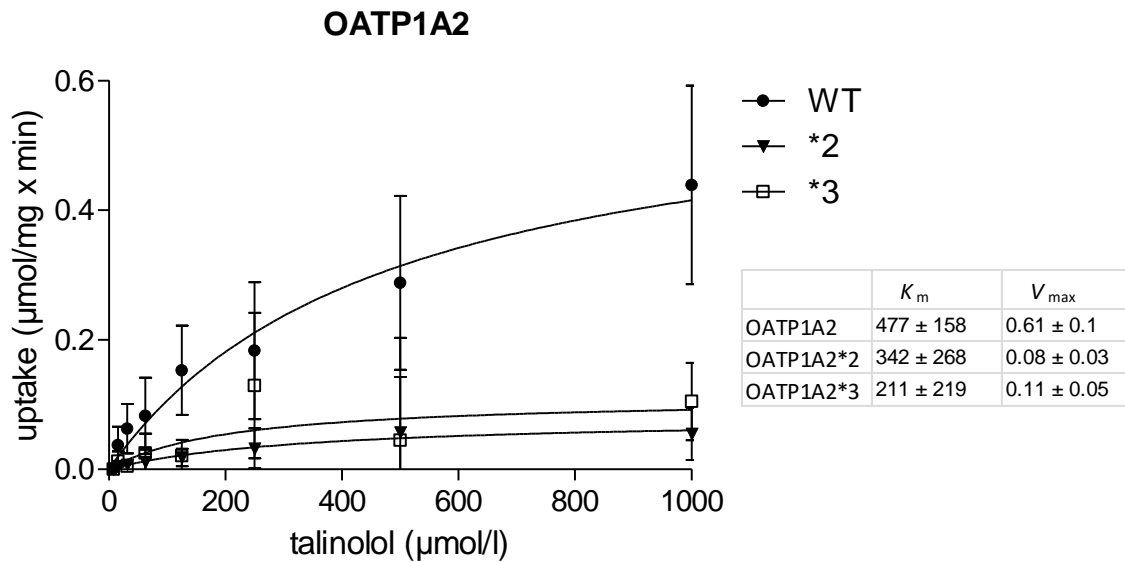
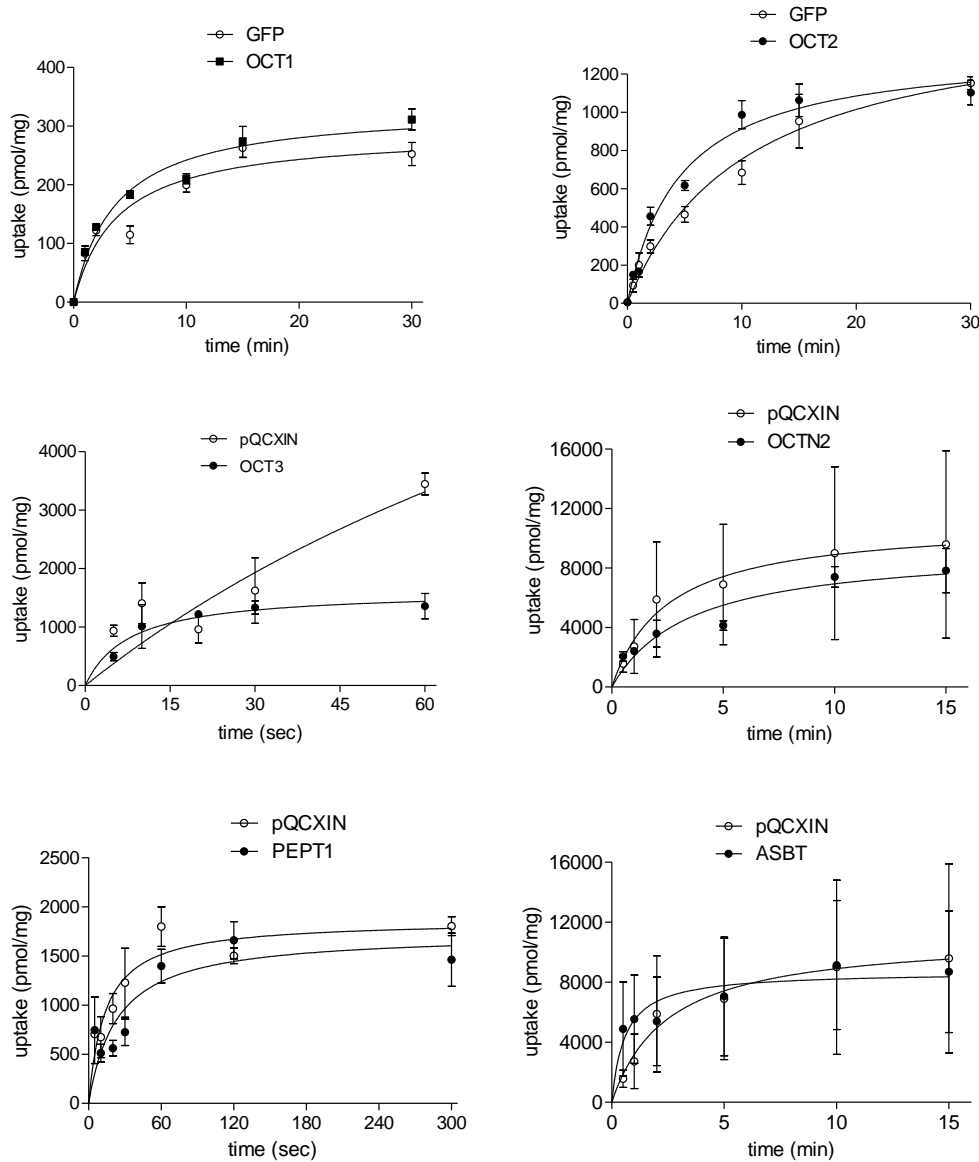


Figure 5: Concentration dependent net-uptake ($M \pm SD$) of talinolol (- control) in stably transfected HEK293 cells expressing OATP1A2 and its genetic variants OATP1A2 *2 and *3.



*GFP / pQCXIN: control vectors

Figure 6: Time dependent uptake ($M \pm SD$) of talinolol in stably transfected MDCK2 cells expressing OCT1, OCT2, OCT3, OCTN2 and PEPT1 and in stably transfected HEK293 cells expressing ASBT.

5.2. Affinity of talinolol to efflux transporter proteins:

ATP dependent transport assays were conducted with four efflux transporters, namely P-glycoprotein (P-gp) and Multidrug resistance protein 1, 2 and 3 (MRP1 – 3). For these purpose we used inside-out lipovesicles prepared from cells, stably transfected with P-gp, MRP1, MRP2 or MRP3.

Only P-gp and MRP3 but not MRP1 or MRP2 could show a time a ATP dependent transport of talinolol (Figure 7).

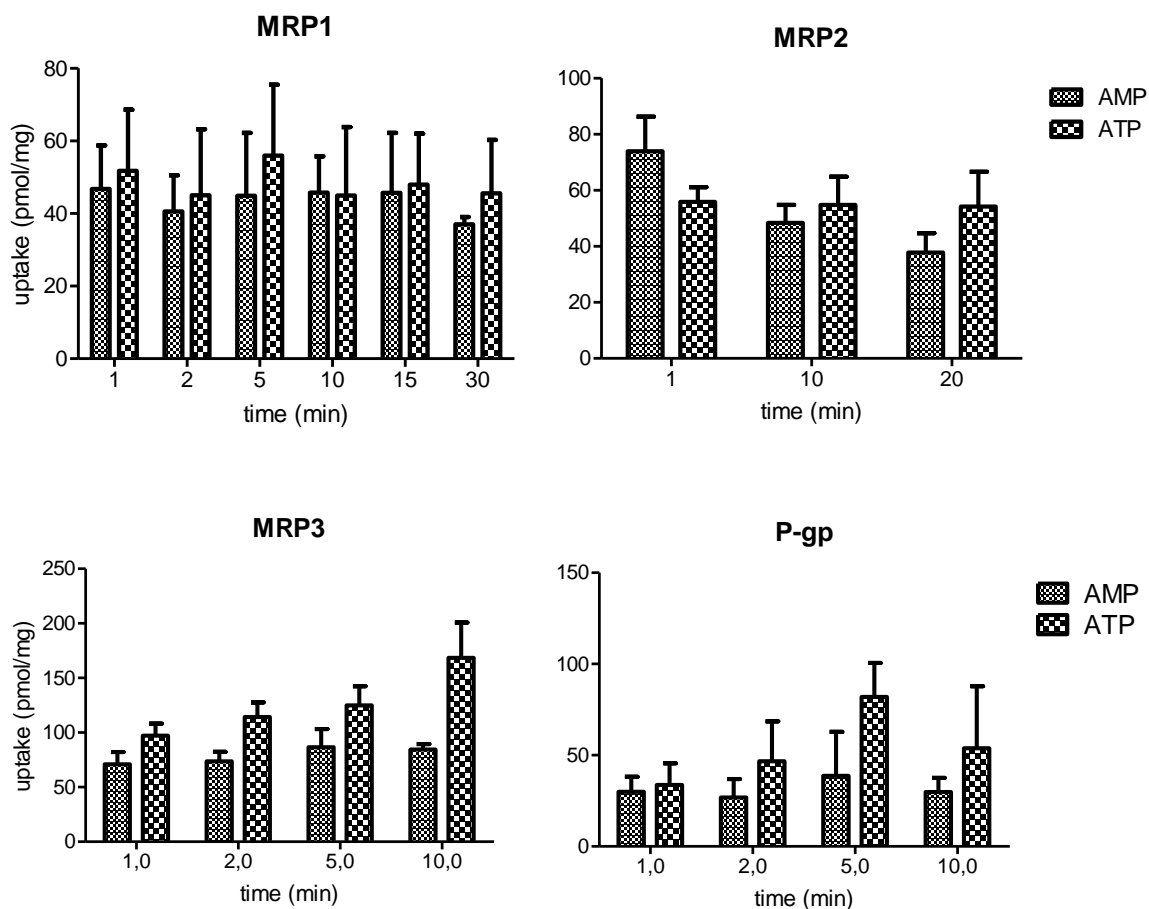


Figure 7: Time and ATP dependent transport ($M \pm SD$) of talinolol in lipovesicles expressing MRP1, MRP2, MRP3 and P-gp.

In a further step, we conducted concentration dependent assays with the inside-out lipovesicles expressing P-gp and MRP3, in order to measure their affinity and capacity for talinolol. Here again, the transport of talinolol was confirmed through both transport proteins (Figure 8), with P-gp having K_m of $175 \pm 206 \mu\text{mol/l}$, V_{max} of $14 \pm 10.8 \mu\text{mol/mg}\times\text{min}$ and $C_{\text{int.}}$ of $0.08 \pm 0.11 \text{ ml}/\mu\text{g}\times\text{min}$ and MRP3 having K_m of $86.8 \pm 62.8 \mu\text{mol/l}$ and V_{max} of $133 \pm 51.5 \mu\text{mol/mg}\times\text{min}$ and $C_{\text{int.}}$ of $1.53 \pm 1.26 \text{ ml}/\mu\text{g}\times\text{min}$ (Table 5).

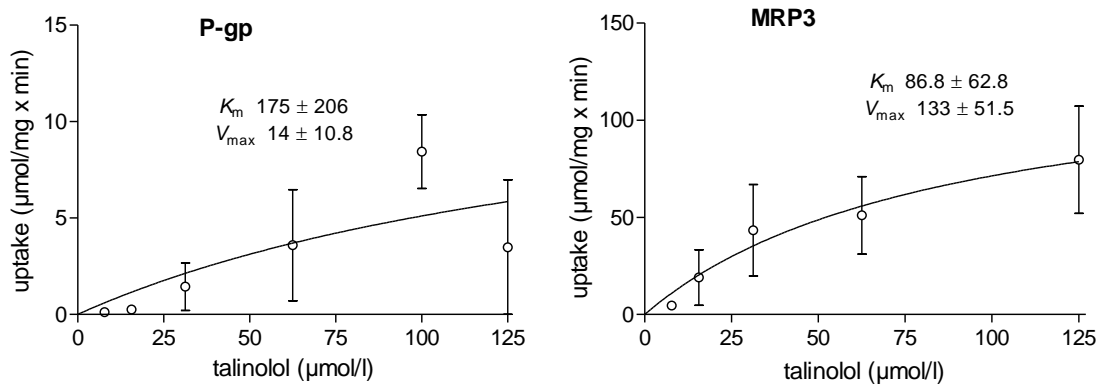


Figure 8: Concentration dependent net- transport ($M \pm SD$) of talinolol (- control) in lipovesicles expressing P-gp and MRP3.

Table 5: Talinolol uptake in inside-out lipovesicle expressing P-gp and MRP3.

Cell line/ Transporter	K_m ($\mu\text{mol/l}$)	V_{max} ($\mu\text{mol/mg}\times\text{min}$)	$C_{\text{int.}}$ ($\text{ml}/\mu\text{g}\times\text{min}$)
P-gp	175 ± 206	14 ± 10.8	0.08 ± 0.11
MRP3	86.8 ± 62.8	133 ± 51.5	1.53 ± 1.26

5.3. Retrospective pharmacogenomic results:

As described above, genotyping was conducted in a total of 39 subjects to investigate the potential effect of the different genetic variants on the pharmacokinetics of talinolol represented by the main parameters AUC, F (when available), C_{max} , CL and $T_{1/2}$. Genotyping results are summarized in Table 6.

Table 6: Summarized results of the retrospective genotyping.

Transporter	SNP	genotype	Number of subjects	
			Talinolol p.o.	Talinolol i.v.
OCT1	rs72552763 c.1260_1262 delGAT	GAT/GAT	22	12
		del/GAT	12	5
		del/del	1	1
	rs55918055 c.262T>C	T/T	34	20
		T/C	0	0
		C/C	0	0
	rs12208357 c.181C>T	C/C	29	12
		C/T	5	4
		T/T	0	0
OATP1A2	rs10841795 c.38T>C	T/T	23	13
		T/C	12	6
		C/C	1	1
	rs11568563 c.516A>C	A/A	32	14
		A/C	4	4
		C/C	0	0
OATP1B1	rs4149056 c.521T>C	T/T	26	14
		T/C	10	5
		C/C	0	0
	rs2306283 c.388A>G	A/A	10	5
		A/G	21	12
		G/G	5	2

Transporter	SNP	genotype	Number of subjects		
			Talinolol p.o.	Talinolol i.v.	
OATP1B3	rs4149117	T/T	3	2	
	c.334T>G	T/G	9	3	
		G/G	18	8	
MRP2	rs717620	C/C	22	13	
		C/T	13	5	
		T/T	3	2	
	rs8187710	G/G	33	16	
		G/A	4	3	
		A/A	0	0	
rs2273697	G/G	25	13		
	G/A	13	8		
	A/A	1	1		
rs3740066	C/C	13	7		
	c.3972C>T	C/T	16	8	
		T/T	9	5	
ABCC3	rs4793665	C/C	9	4	
		C/T	15	9	
		T/T	14	7	
P-gp	rs2032582c	C/C	11	5	
		C/T	16	12	
		T/T	10	2	
	rs1128503	C/C	11	4	
		c.1236 C>T	C/T	19	14
			T/T	9	3
	rs1045642	C/C	12	6	
		c.3435 C>T	C/T	20	11
			T/T	6	3

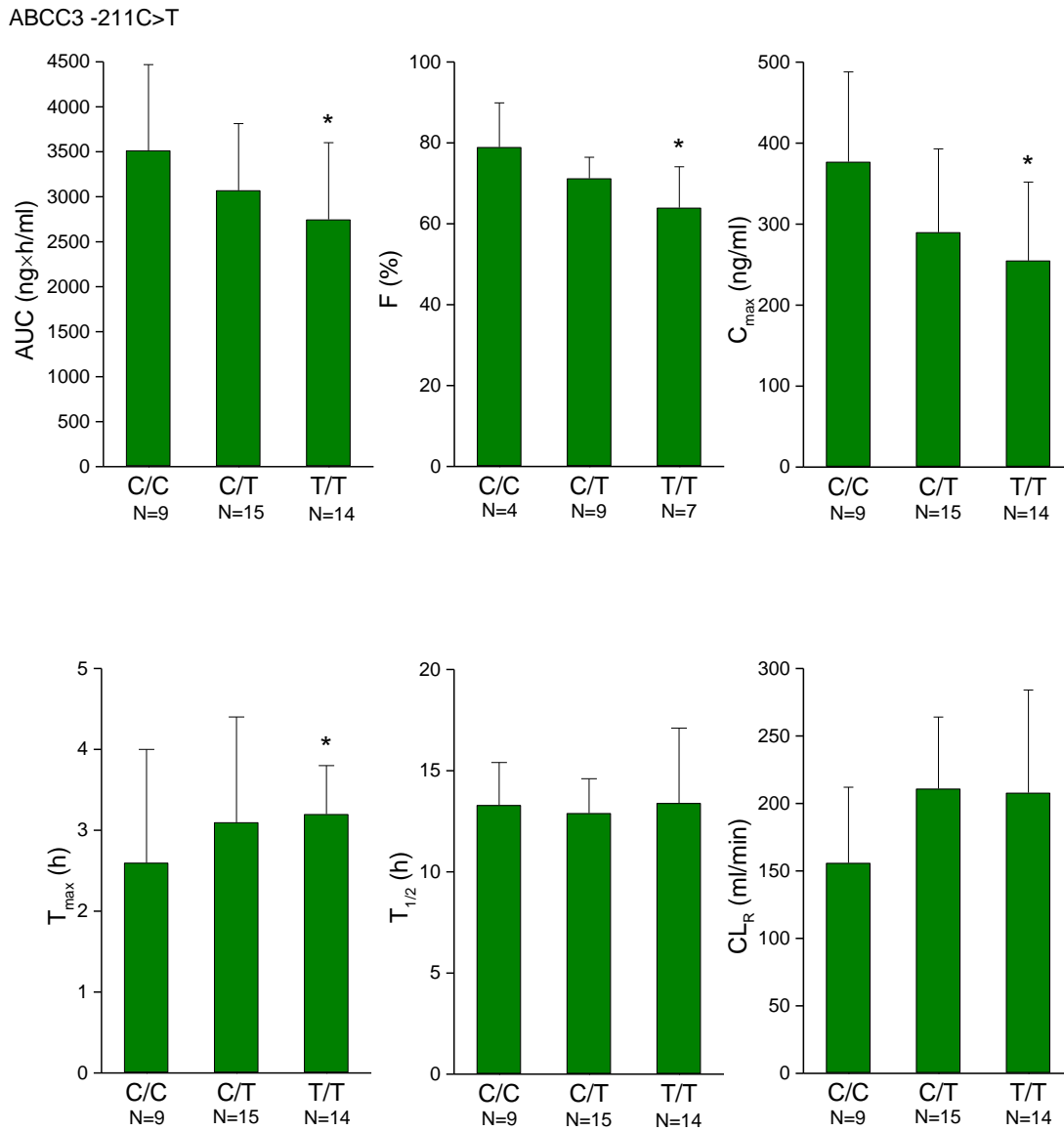
Regarding the uptake transport proteins OCT1 (Figure S1-2), OATP1A2 (Figure S3-4), OATP1B1 (Figure S5-6) and OATP1B3 (Figure S7) and the efflux transport protein MRP2 (Figure S8-11), none of the studied genetic polymorphisms could be correlated with a significant change in any of the pharmacokinetic parameters calculated for talinolol (see appendix: Figure S1-11, Table S1). However, it is worth mentioning that the studied subject numbers were relatively low for these polymorphisms or even for the wild type.

On the other hand and when studying the genetic variant *ABCC3* -211C>T, a significant correlation was found with the pharmacokinetics of talinolol as subjects who are homozygote for *ABCC3* -211T had significantly lower AUC, F and C_{max} and higher T_{max} comparing with the subjects carrying the wild-type *ABCC3*, whereas no significant difference was seen regarding $T_{1/2}$ and CL_R (Figure 9, Table 7).

Furthermore, and in order to test the practical relevance of this variant, we compared the individuals carrying the homozygote variant with the rest of the study population. Here again, -211T was correlated with a lower absorption but not elimination of talinolol represented by significantly decreased AUC, F and C_{max} in the individuals with the homozygote variant (Figure 10, Table 7).

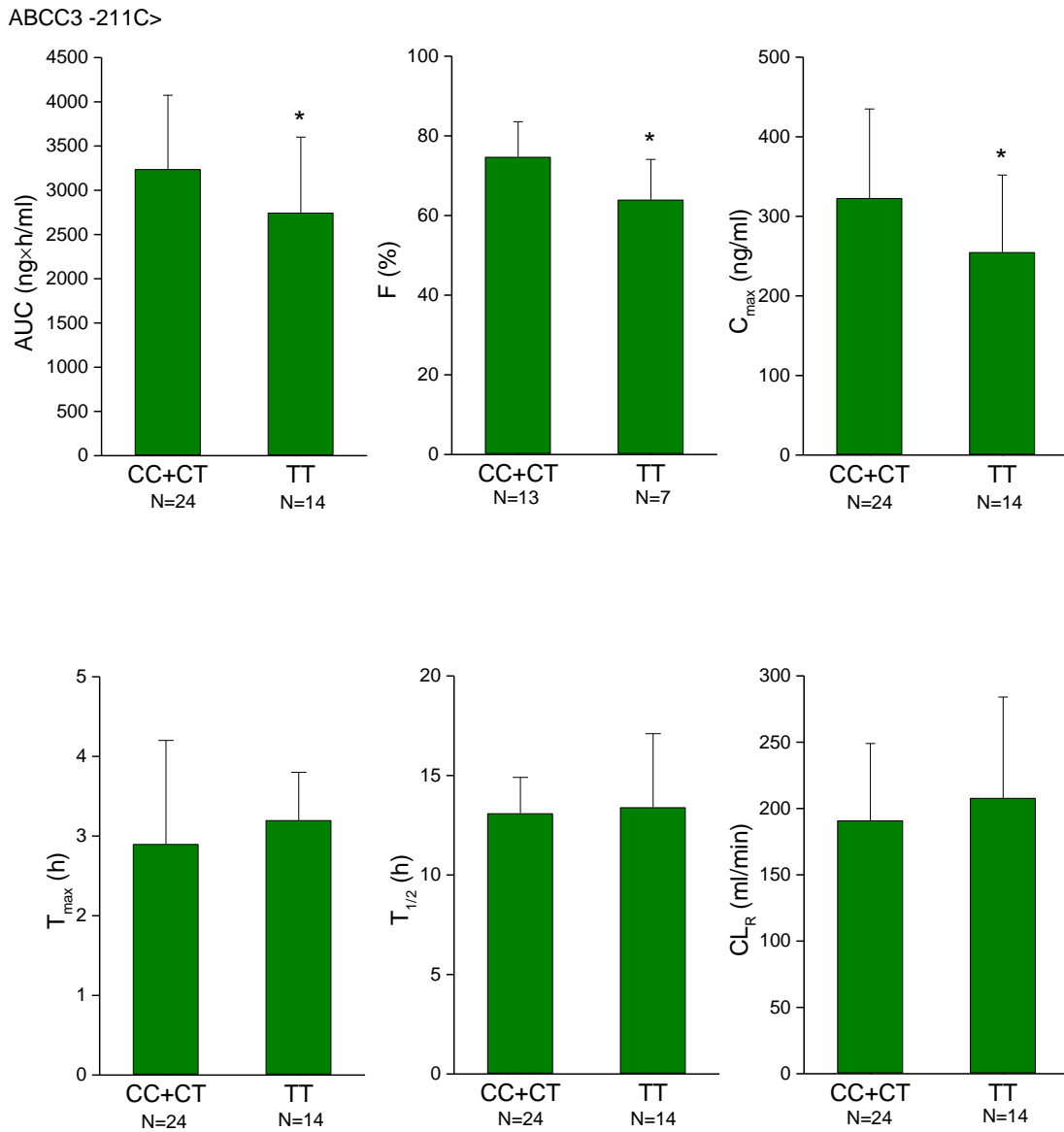
Table 7: Pharmacokinetic characteristics (mean \pm SD) of talinolol after single dose oral administration of 100 mg talinolol in respect of the *ABCC3* -211C>T polymorphism.

Genotype	AUC (ngxh/ml)	F (%)	C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	CL_R (ml/min)
C/C	3516 \pm 952	79 \pm 11	377 \pm 111	2.6 \pm 1.4	13.3 \pm 2.1	156 \pm 56
C/T	3072 \pm 741	71 \pm 5	290 \pm 103	3.1 \pm 1.3	12.9 \pm 1.7	211 \pm 53
C/C + C/T	3239 \pm 835	75 \pm 9	323 \pm 112	2.9 \pm 1.4	13.1 \pm 1.8	191 \pm 59
T/T	2747 \pm 854	64 \pm 10	255 \pm 97	3.2 \pm 0.6	13.4 \pm 3.7	209 \pm 76



* : P< 0.05 vs. C/C

Figure 9: AUC, F, C_{max}, T_{max}, T_{1/2} and CL_R of talinolol after single dose oral administration 100 mg talinolol in respect of the ABCC3 -211C>T polymorphism.



* : P < 0.05 vs. C/C + C/T

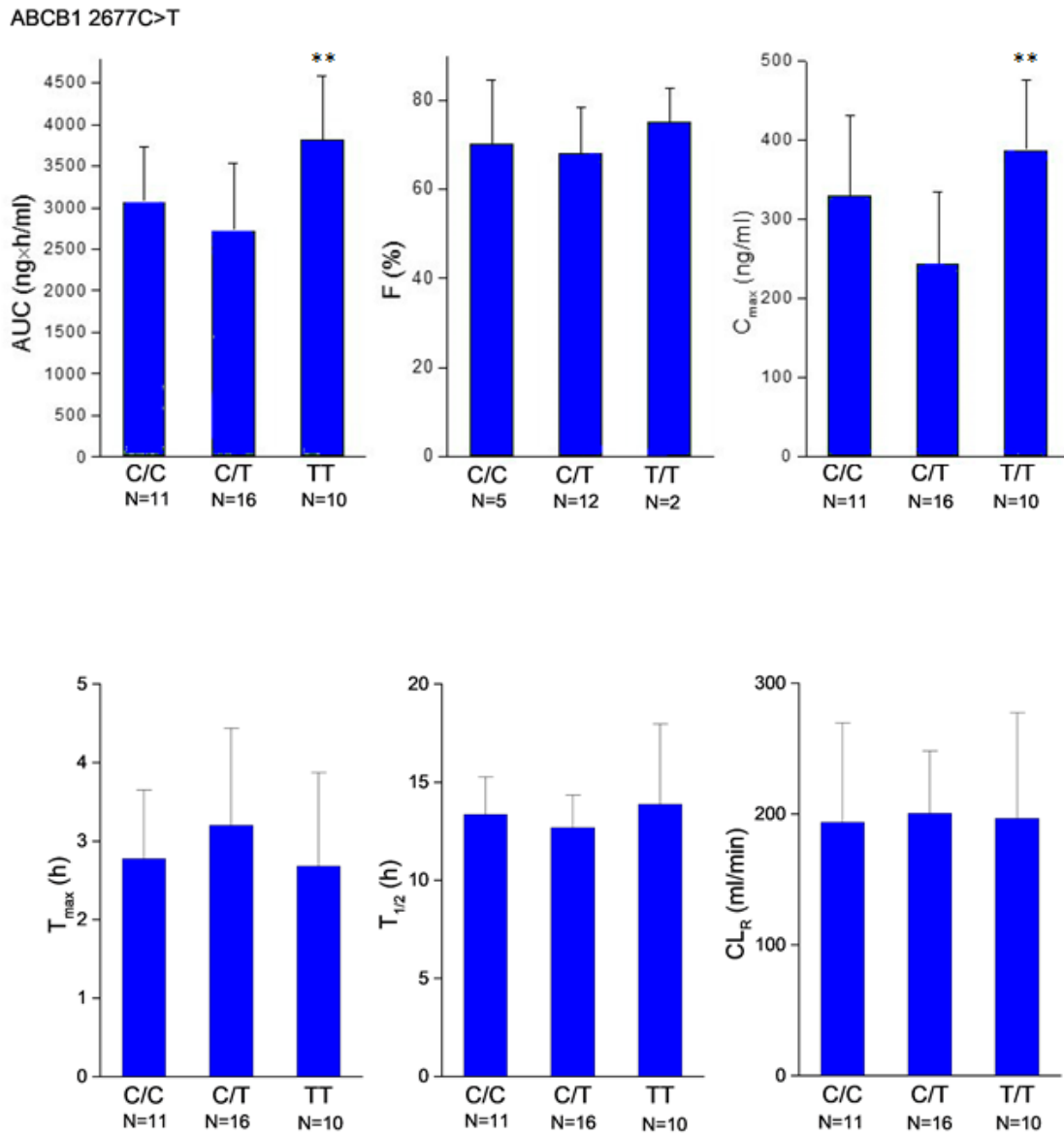
Figure 10: AUC, F, C_{max}, T_{max}, T_{1/2} and CL_R CL_R of talinolol after single dose oral administration 100 mg talinolol in respect of the ABCC3 -211C>T polymorphism.

For P-gp, the effect of the genetic polymorphisms c.2677G>T (rs2032582), c.1236C>T (rs1128503) and c.3435C>T (rs1045642) was studied regarding the pharmacokinetics of talinolol. In our subject pool, these variants had allele frequencies of 48%, 47% and 42%, respectively, corresponding with the frequencies described in the NCBI- dbSNP database (47%, 45% and 54%, respectively).

Our analysis has shown a significant increase in AUC and C_{max} in subjects carrying the homozygote 2677T gene, while other parameters were not significantly changed (Figure 11, Table 8).

Table 8: Pharmacokinetic characteristics (mean \pm SD) of talinolol after single dose oral administration of 100 mg talinolol in respect of the *ABCB1* 2677C>T, 1236C>T and 3435C>T polymorphisms.

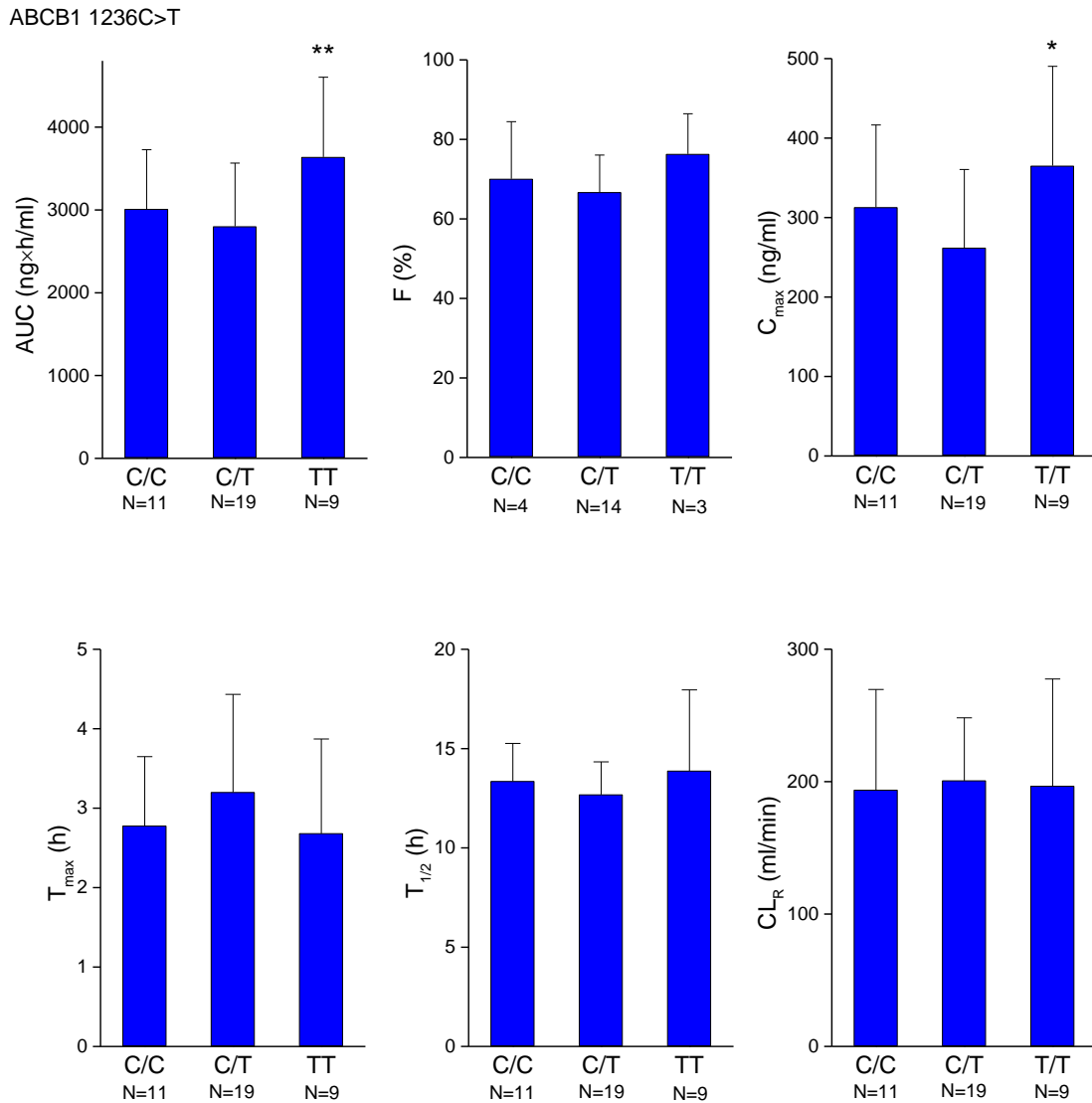
Genetic variant	genotype	AUC (ng×h/ml)	F (%)	C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	CL_{ren} (ml/min)
<i>ABCB1</i> 2677C>T	C/C	3069 \pm 664	70 \pm 14	330 \pm 102	2.7 \pm 0.8	13 \pm 1.8	194 \pm 76
	C/T	2726 \pm 805	68 \pm 10	243 \pm 91	3.2 \pm 1.3	12 \pm 1.6	205 \pm 49
	T/T	3739 \pm 707	74 \pm 7	372 \pm 84	3 \pm 1.2	13 \pm 2.1	193 \pm 76
<i>ABCB1</i> 1236C>T	C/C	3011 \pm 715	70 \pm 14	313 \pm 104	2.8 \pm 0.9	13.4 \pm 1.9	194 \pm 76
	C/T	2801 \pm 765	67 \pm 9	262 \pm 99	3.2 \pm 1.2	12.7 \pm 1.7	201 \pm 47
	T/T	3640 \pm 962	76 \pm 5	365 \pm 125	3 \pm 1.2	13.9 \pm 4.1	197 \pm 81
<i>ABCB1</i> 3435C>T	C/C	2945 \pm 609	66 \pm 12	303 \pm 84	2.7 \pm 0.8	13.4 \pm 1.8	194 \pm 76
	C/T	2809 \pm 623	70 \pm 12	336 \pm 118	3.2 \pm 1.3	12.4 \pm 1.6	205 \pm 49
	T/T	3530 \pm 1146	76 \pm 6	298 \pm 111	2.8 \pm 1.2	12.5 \pm 2	199 \pm 80



** : P< 0.005 vs. C/T

Figure 11: AUC, F, C_{max}, T_{max}, T_{1/2} and CL_R of talinolol after single dose oral administration 100 mg talinolol in respect of the *ABCB1* 2677C>T polymorphism.

Regarding the 1236C>T polymorphism, the analysis showed an increased AUC and C_{max} in subjects carrying the homozygote 1236T gene, while no significant effect was noticed when it comes to the other parameters (Figure 12, Table 8).



* : $P < 0.05$ vs. C/T , ** : $P < 0.005$ vs. C/T

Figure 11: AUC, F, C_{max} , T_{max} , $T_{1/2}$ and CL_R of talinolol after single dose oral administration 100 mg talinolol in respect of the *ABCB1* 1236C>T polymorphism.

The polymorphism 3435C>T was also found to influence the plasma concentration of talinolol, as the subjects homozygote for 3435T had a higher AUC of talinolol comparing with other subjects. No significant change was seen in the other parameters (Figure 12, Table 8)

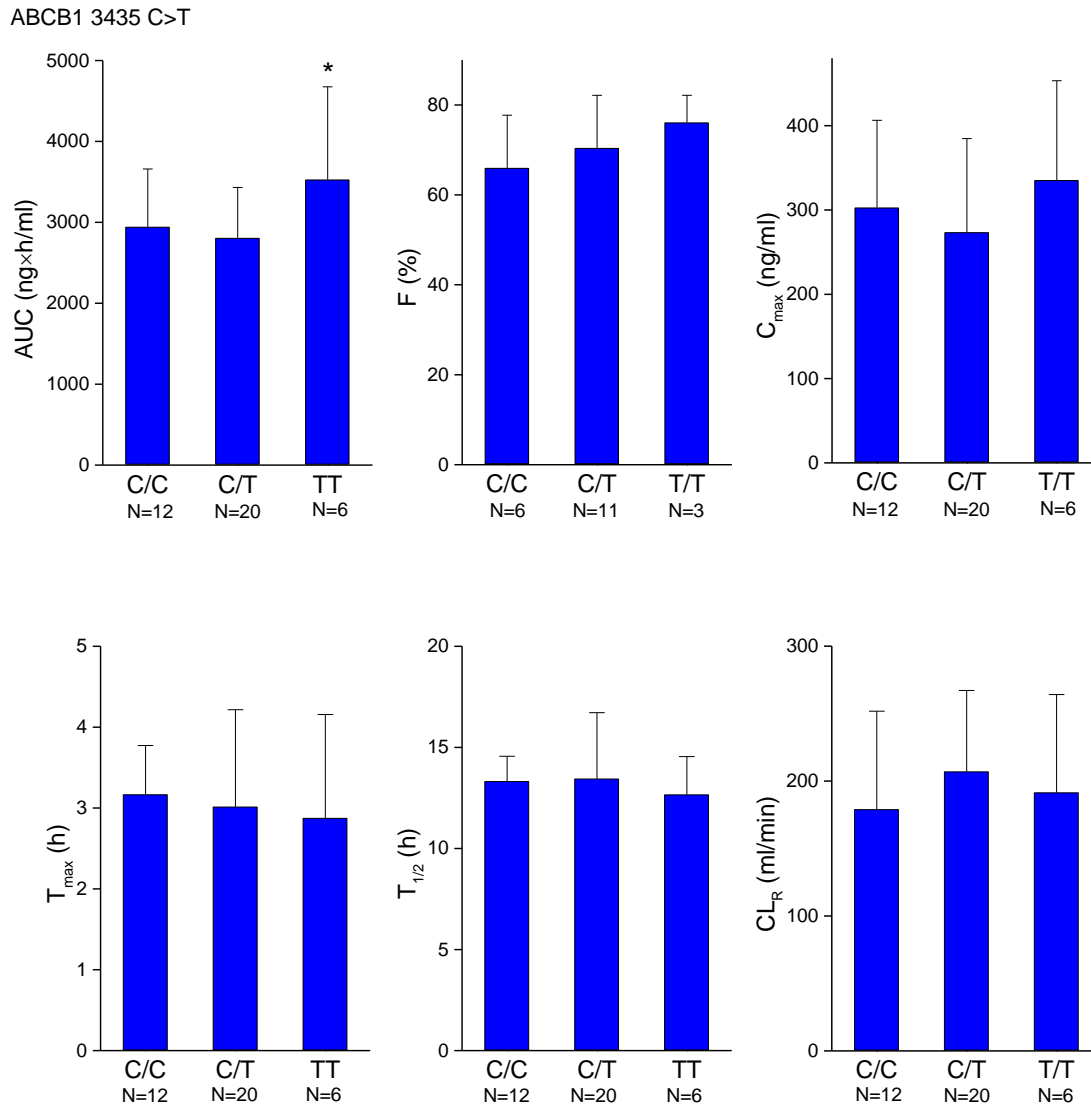


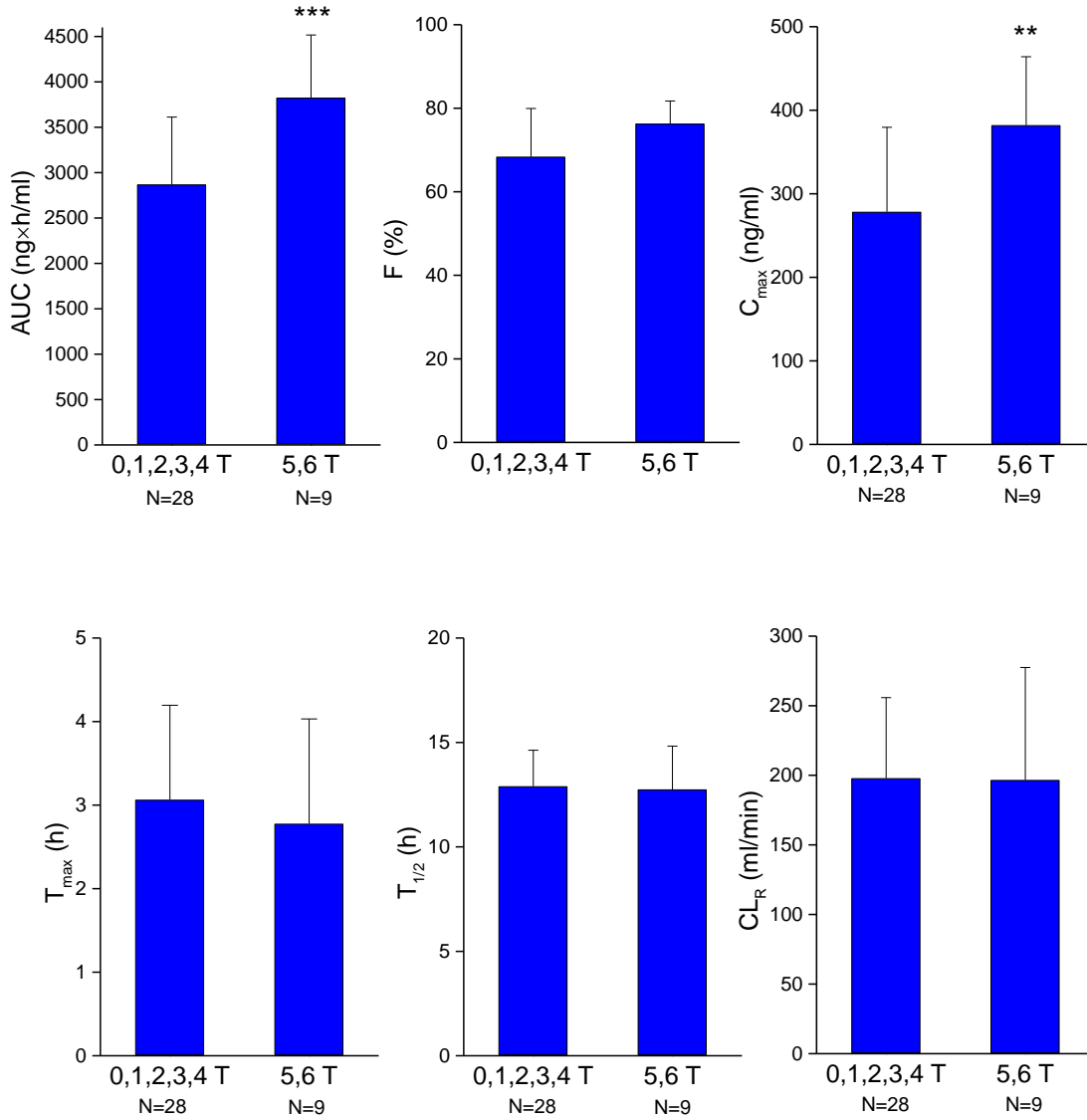
Figure 12: AUC, F, C_{max}, T_{max}, T_{1/2} and CL_R of talinolol after single dose oral administration 100 mg talinolol in respect of the *ABCB1* 3435 C>T polymorphism.

The three most common coding SNPs at nucleotides 1236, 2677, and 3435 are observed most frequently as either the 893Ala-containing CGC haplotype or 893Ser-containing TTT haplotype in most ethnic groups.^{55,56} However, the 893Ser-bearing TTT (1236, 2677, 3435) haplotype was found to be associated with an increased digoxin exposure.⁵⁷ For these reasons, we have also assessed the effect of the three genes- haplotype on talinolol pharmacokinetics. The analysis was shown that subjects, homozygote for at least two variants with being hetro- or homozygote for the third one (5, 6 T), had a significant higher AUC and C_{max} than the rest (0, 1, 2, 3, 4 T), whereas the difference in other parameters was not significant (Figure 13, Table 9).

Table 9: Pharmacokinetic characteristics (mean \pm SD) of talinolol after single dose oral administration of 100 mg talinolol in respect of the *ABCB1* 1236-2677-3435-TTT haplotype.

Genotype	AUC (ngxh/ml)	F (%)	C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	CL_R (ml/min)
0,1,2,3,4 T	2869 \pm 743	68 \pm 12	278 \pm 101	3.1 \pm 1.1	12.9 \pm 1.7	198 \pm 58
5,6 T	3826 \pm 690	76 \pm 5	382 \pm 82	2.8 \pm 1.3	12.8 \pm 2.1	197 \pm 81

ABCB1 1236-2677-3435 TTT haplotype



** : P< 0.005 vs. 0,1,2,3,4T , *** : P< 0.0005 vs. 0,1,2,3,4T

Figure 13: AUC, F, C_{max}, T_{max}, T_{1/2} and CL_R of talinolol after single dose oral administration 100 mg talinolol in respect of the *ABCB1* 1236-2677-3435 TTT haplotype.

As presented before, the studied genetic variants of both *ABCB1* and *ABCC3* were seen to significantly influence the *in-vivo* pharmacokinetics of talinolol which opens the door for a possible interplay between these two transporters, especially since both of them are believed to be expressed in the human liver and intestine. Therefore, we have compared talinolol pharmacokinetics between two groups of subjects:

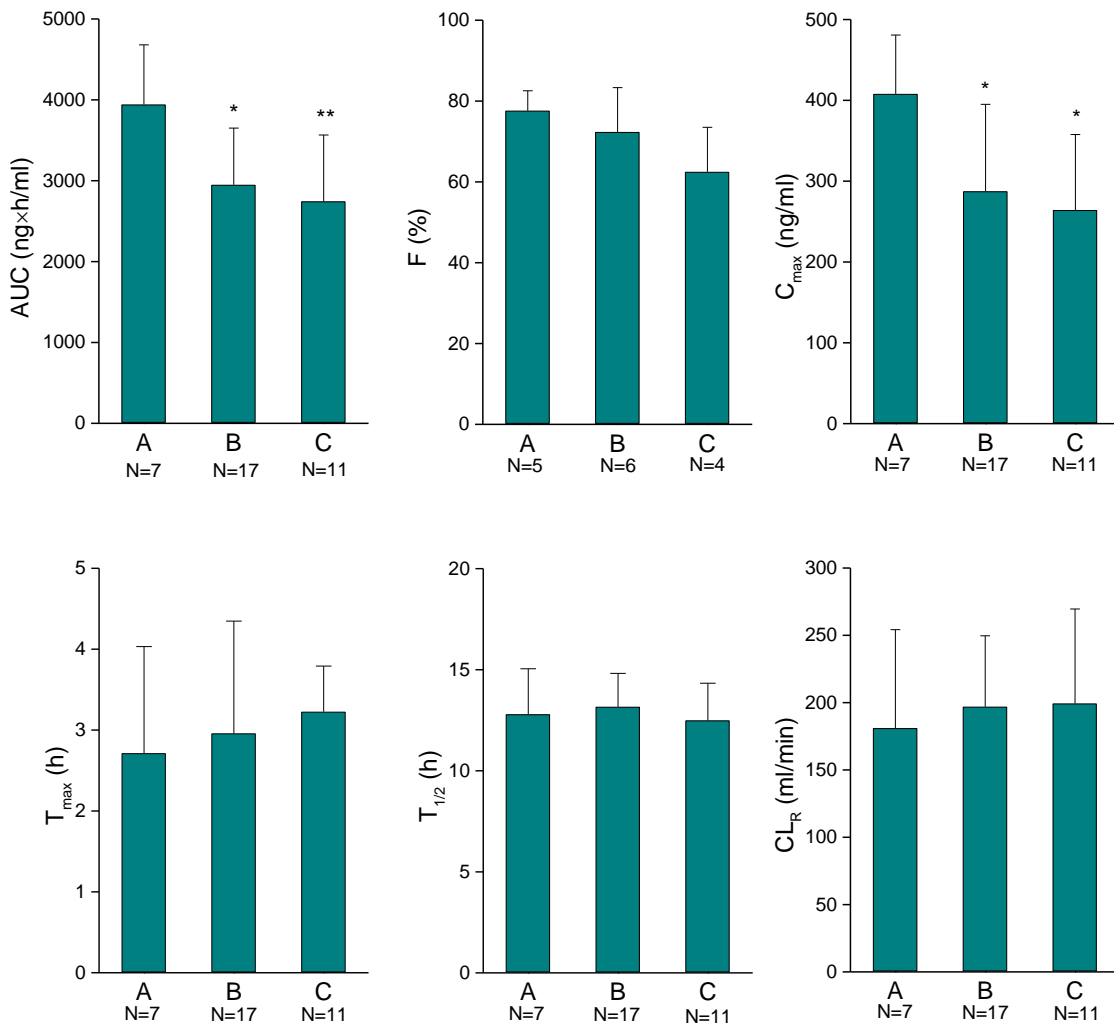
Group A (deficient P-gp and normal MRP3): included subjects with 5 or 6 T- alleles in the haplotype *ABCB1* 1236-2677-3435 and who were not homozygous for *ABCC3* -211T (had CC or CT).

Group B (normal P-gp and MRP3): included subjects with not more than 4 T- alleles in the haplotype *ABCB1* 1236-2677-3435 and who were not homozygous for *ABCC3* -211T (had CC or CT).

Group C (normal P-gp and deficient MRP3): included subjects with no more than 4 T- alleles in the haplotype *ABCB1* 1236-2677-3435 and who were homozygous for *ABCC3* -211T.

Corresponding with the previous pharmacogenomic results, the subjects in group A seemed to have significantly higher AUC and C_{max} of talinolol comparing with those in group B (Figure 14, Table 10). Furthermore, the differences between the mean AUC values and between the mean C_{max} values in these two groups were the highest in the whole analysis with AUC increased by 43% and C_{max} by 55% in group A. On the other hand, F also increased by 24% in group A, and even though this increase wasn't statically significant, applying the Jonckheere- Terpstra-Test showed us a significantly positive trends for all three parameters (AUC, F and C_{max}). As expected, no significant change was seen in $T_{1/2}$ or CL_R .

ABCB1 1236-2677-3435 TTT haplotype and ABCC3 -211T>C



* : P< 0.05 vs. A / **: P< 0.01 vs. A

A: deficient P-gp and normal MRP3

B: normal P-gp and MRP3

C: normal P-gp and deficient MRP3

Figure 14: AUC, F, C_{max}, T_{max}, T_{1/2} and CL_R of talinolol after single dose oral administration 100 mg talinolol in respect of ABCB1 1236-2677-3435 TTT haplotype and ABCC3 -211T>C polymorphisms.

Table 10: Pharmacokinetic characteristics (mean \pm SD) of talinolol after single dose oral administration of 100 mg talinolol in respect of the *ABCC3* - 211C>T polymorphism and the *ABCB1* 1236-2677-3435 TTT haplotype.

Genotype group	<i>AUC</i> (ng×h/ml)	<i>F</i> (%)	<i>C</i> _{max} (ng/ml)	<i>T</i> _{max} (h)	<i>T</i> _{1/2} (h)	<i>CL</i> _R (ml/min)
A	3942 \pm 738	78 \pm 5	408 \pm 73	2.7 \pm 1.3	12.8 \pm 2.3	181 \pm 73
B	2949 \pm 702	72.3 \pm 11	288 \pm 108	2.96 \pm 1.39	13.2 \pm 1.65	197 \pm 52.8
C	2745 \pm 820	63 \pm 11	264 \pm 93	3.2 \pm 0.5	12.5 \pm 1.8	200 \pm 70

6. Discussion:

The presented research was aimed to expand our knowledge regarding the pharmacokinetics of the β_1 -blocker talinolol and to add additional evidence for its suitability as a probe drug for the efflux transporters P-gp by evaluation of mechanisms that enable vectorial transport in the human intestine.

Our *in-vitro* experiments showed that talinolol is a substrate of uptake transporters from the OATP family, namely OATP 1A2, 2B1, 1B1 and 1B3, and of the hepatic transporter NTCP. However, talinolol was not a substrate of any other uptake transporter evaluated in the study as OCT1, OCT2, OCT3, OCTN2 and PEPT1. Furthermore, the genetic variants of both OATP2B1 and OATP1A2 were shown to influence the *in-vitro* transport of talinolol. In case of OATP2B1, the three variants (V201M, R312Q and S486F) were gain-of-function proteins compared to the wildtype with V201M to be associated with the highest capacity but lowest affinity for talinolol. Regarding OATP1A2, both variants (*2 and *3) showed significant loss of function compared to the wildtype transport protein.

Talinolol was also a substrate for the efflux carriers P-gp and MRP3 but not for MRP1 and MRP2. These results correspond with the available evidence that talinolol is a substrate of P-gp, but disagrees with the results of a former publication which showed that talinolol is a substrate of MRP2 because it was better absorbed and distributed in *Abcb2* deficient rats.⁶⁶ However, the reason for the discrepancy can be well understood by considering a recent study, which found other transport proteins including P-gp and *Mrp3* to be overexpressed in *Mrp2* deficient rats.⁵⁵

In summary, the *in-vitro* data on membrane transport of talinolol as obtained using the cell-platform of the C_DAT and by using transport kinetic characteristics (K_m , V_{max} , CL_{int}) after correction of transport activity to the specific cellular transporter protein abundance, enable a new and profound discussion of the mechanisms for intestinal absorption, distribution and elimination of talinolol in man (Figure 20). Furthermore, the well-known drug-drug interactions with inducers and inhibitors of drug transporters for talinolol can be discussed in more detail and in a new light

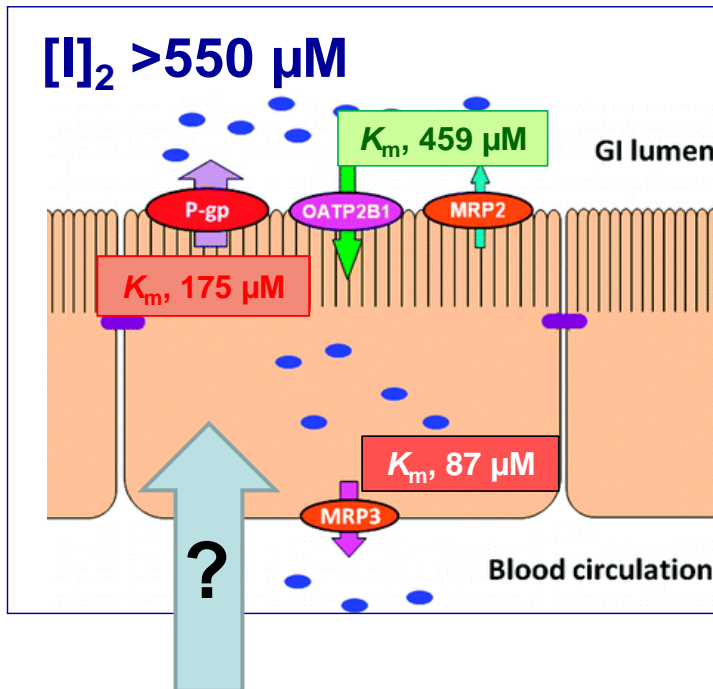
that consider the complexity of the processes involved in membrane transfer of the probe drug.

Intestinal absorption:

Talinolol is slowly and incompletely absorbed after oral administration, despite the absence of significant first pass metabolism.^{10,11,13,15} Furthermore, its absorption from the intestine is known to be regio-selective as confirmed by the data on systemic availability of talinolol simultaneously measured by the triple lumen tube perfusion technique.^{27,28} Both facts confirmed the role of the intestinal efflux transporter P-gp, which is known to decrease the bioavailability of any substrate and to be regio-selectively expressed along the human small and large intestine.^{32,33} However, the mechanisms by which talinolol is transported through the apical and basolateral cell membrane still needed to be explained previously.

Our results clearly demonstrate that the apically localized OATP2B1 might be involved in intestinal uptake of talinolol as the K_m for OATP2B1 was lower than the concentration of talinolol in the small intestine after oral administration of a therapeutic dose ($[I]_2 = 550 \mu\text{mol/l}$ considering a dose of 50 mg taken with 240 ml water). This finding can explain, at least in part, why talinolol is less absorbed when administered together with P-gp inhibitors like verapamil or grapefruit juice^{26,56}, because both substances are known to inhibit transporters of the OATP family.⁵⁷ Inhibition of talinolol uptake by verapamil and grapefruit juice obviously overshadows the effect on bioavailability which is expected by inhibition of P-gp due to the increase of exposure. Regarding the affinity of talinolol to the uptake transporter OATP1A2 *in-vitro*, recent studies could not detect relevant expression levels in any intestinal segment which excludes a significant role of OATP1A2 in the absorption of talinolol.^{51,58}

The MRP3 efflux transporter, which was localized to the basolateral membrane of the enterocytes⁵⁸, seems to play a determinant role in intestinal absorption of talinolol, especially as it has a higher affinity and a higher capacity than P-gp. Therefore, talinolol absorption seems to result from interplay of at least the three transporters P-gp, OATP2B1 and MRP3 (Figure 15).

**Vectorial transfer**

OATP2B1: apical, low-affine uptake

P-gp: apical, high-affine efflux

MRP3: basal, high-affine efflux

Figure 15: The vectorial transport of talinolol in the human intestine as a result of the interplay among OATP2B1, P-gp and MRP3.

The conception on the mechanisms of intestinal talinolol absorption was confirmed by the data of our retrospective pharmacogenomics analysis of the pharmacokinetic results of four former clinical studies with talinolol in healthy subjects, which demonstrated a significant effect of the promoter polymorphism of MRP3 (-211C>T) (Figure 9 &10) and specific haplotypes of *ABCB1* which are known to influence the P-gp transport function (Figure 13). This analysis has shown that subjects with wildtype MRP3 function (carrying at least one *ABCC3* -211C) but presumably lowest P-gp function (5 or 6 T- alleles in the haplotype *ABCB1* 1236-/2677/-3435) had the best bioavailability of talinolol and subjects with the opposite genetic constellation had the worst extent of absorption (Figure 14).

Apart from that, we are still unable to explain the mechanism through which talinolol is being secreted from the blood stream into the gut lumen.²⁸ This

secretion is believed to be mediated by a basolateral uptake transporter which is so far unknown but worth to be discovered.

Entero-hepatic circulation:

It is well known that talinolol undergoes a very minimum metabolism, with less than 1% of its dose being transformed to 4-trans and 3-cis hydroxytalinolol.^{12,13,15,19} Accordingly, the liver may only contribute to talinolol pharmacokinetics through the biliary excretion, which counts for about 10% of talinolol dose given intravenously and results in biliary concentrations up to 90-fold above the serum levels.^{39,67} This may indicate efflux mechanisms with a limited capacity for talinolol.

Our *in-vitro* data suggest that 6 drug transporters may be involved in the hepatic circulation of talinolol, namely P-gp, MRP3, OATP1B1, OATP1B3, OATP2B1 and NTCP. Among the uptake transporters, OATP1B1 had a high affinity but low capacity for talinolol, NTCP had the lowest affinity but the highest capacity while OATP1B3 seemed to have the highest affinity and a relatively high capacity for talinolol. Considering that talinolol enters the hepatocytes through a collectively high capacity high affinity transport, it seems logic to conclude that its biliary elimination results mainly from the interplay between the two efflux transporters, the basolateral located MRP3 and the apical located P-gp (Figure 16). However, this conclusion was supported by our pharmacogenomic analysis, where the genetic polymorphisms of both MRP3 and P-gp had a significant influence on the pharmacokinetics of talinolol. According to our *in-vitro* assays, MRP3 has both higher affinity and higher capacity for talinolol than P-gp, which may explain why only a small percentage is excreted into the bile.

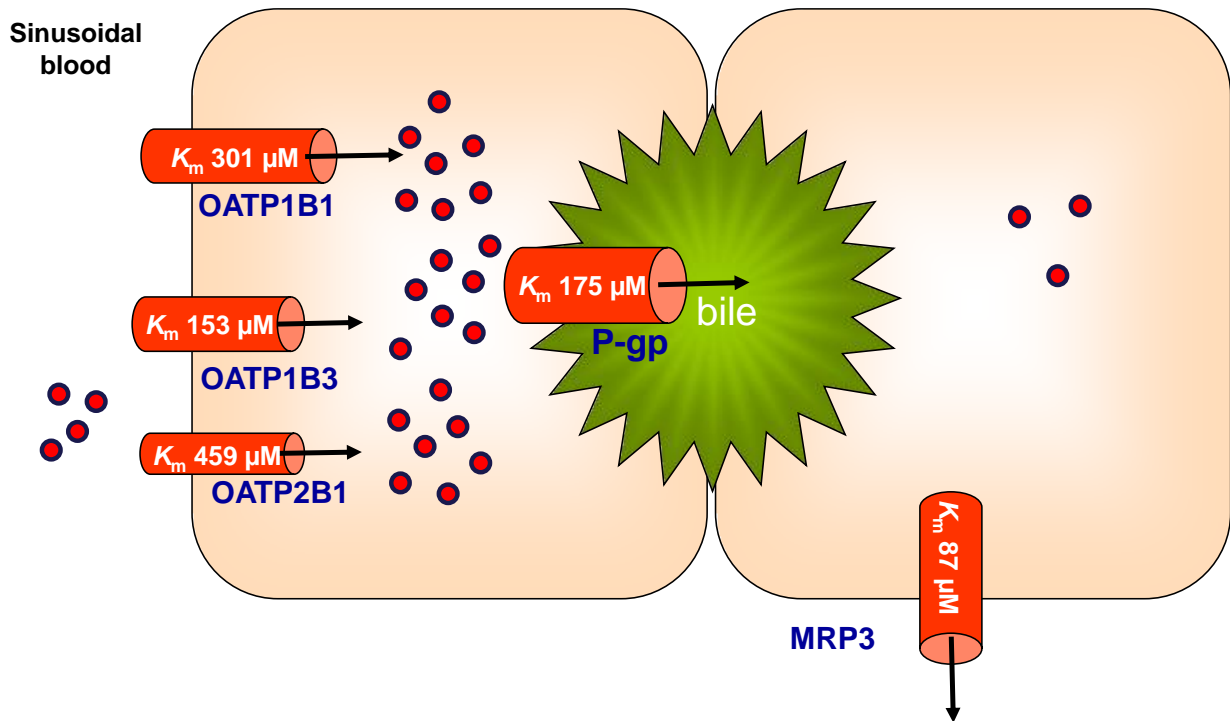


Figure 16: The transport of talinolol through human hepatocytes. This transport results from the interplay among the uptake- (OATP 1B1, 1B3 and 2B1) and the efflux transport proteins (P-gp and MRP3).

Renal clearance:

Concerning its clearance, about 30% of talinolol oral dose is known to undergo renal elimination with a renal clearance about 150–190 ml/min which is higher than the filtration rate in healthy subjects (~120 ml/min).^{10,11,40} This observation can be very well explained by the P-gp mediated active secretion of talinolol. Nevertheless, the uptake mechanism into the tubular cells is still not clear. However, none of the studied P-gp genetic polymorphisms showed significant effects on the renal elimination of talinolol.

Blood-brain barrier:

As talinolol is known to commonly cause some side effects in the central nervous system (e.g. insomnia, depressive mood, nightmares or hallucinations), there must be uptake mechanisms in the brain that overcome the efflux of talinolol as

mediated by P-gp. Our research presented OATP1A2 and OATP2B1 as potential contributors. However, with both transporters having lower affinities and capacities than P-gp, the hypothesis seems less likely, unless these uptake transporters have a higher expression density than P-gp. The presence of other apical or basolateral transporters might be another plausible explanation for the central side effects.

Transporter related interactions:

Considering the previous transport data, it becomes clearer how complex and challenging it is, to predict the drug-drug and food-drug interactions. In regard to talinolol, three drugs are known to cause such complex interactions where several factors are involved:

Erythromycin-talinolol interaction:

In a randomized clinical study, the concomitant administration of a single oral dose of erythromycin increased the oral bioavailability of talinolol significantly (Figure 17). The Authors have suggested that the increase in oral bioavailability of talinolol was caused by increased intestinal net absorption due to Pgp inhibition by erythromycin.⁴⁰ However, in light of our results, we find it essential to consider other possible factors such as:

- The inhibition of hepatic P-gp, OATP1B1 and OATP1B3 which decrease the hepatic uptake and secretion of talinolol.⁵⁹
- The influence of erythromycin on MRP3 and/or any of the uptake transporters involved in the pharmacokinetics of talinolol.
- The prokinetic effect of erythromycin, which accelerates the gastric emptying leading to a water-driven absorption.⁶⁰

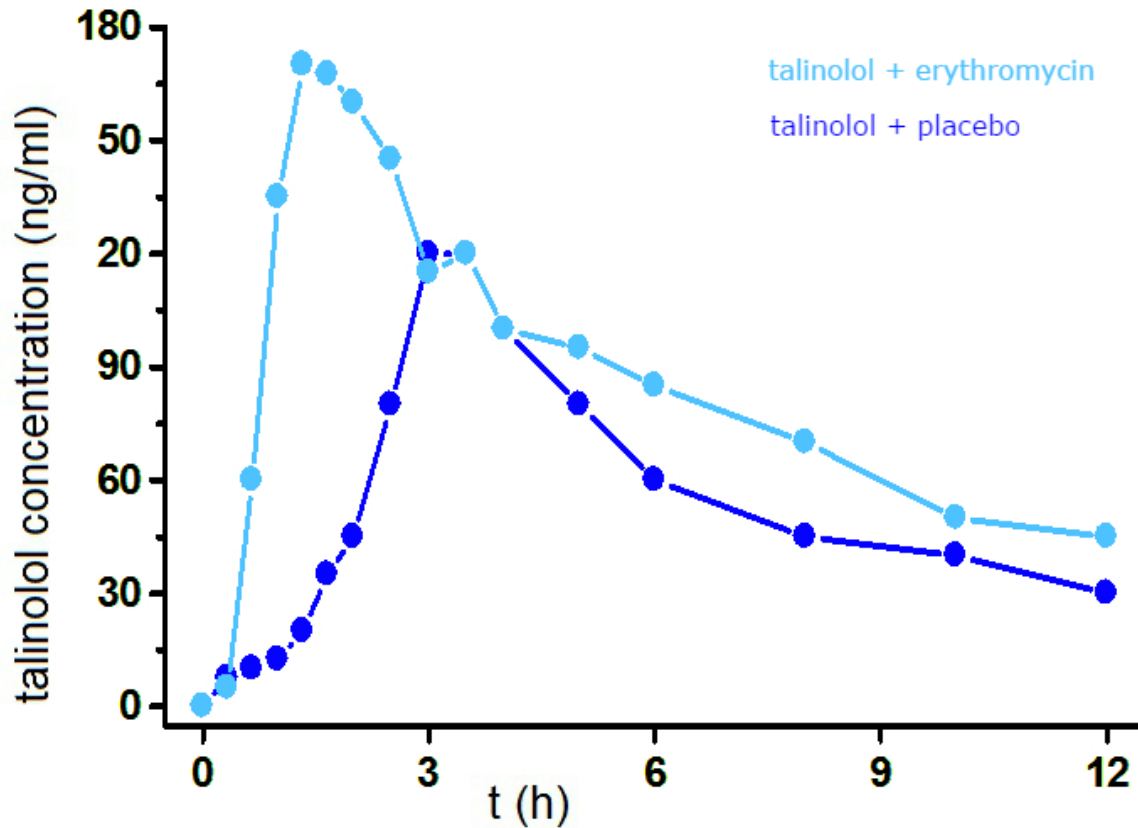


Figure 17: Pharmacokinetics of 50 mg talinolol co-administered with 2 g erythromycin or placebo. The concomitant administration of erythromycin led to a significantly higher absorption of talinolol (modified according to Schwarz et al. 2000).⁴⁰

Grapefruit juice-talinolol interaction:

Grapefruit juice and its constituents are known from *in-vitro* studies to inhibit P-gp efflux transporters.^{25,61} Therefore, it was expected that grapefruit juice would increase the bioavailability of talinolol. Surprisingly, the ingestion of grapefruit juice lowered rather than increased talinolol AUC (Figure 18) which suggested further factors to be involved.⁶² To these factors belong:

- the inhibition of intestinal P-gp which increases the absorption of talinolol
- the inhibition of intestinal OATP2B1 resulting in a decreased uptake
- the caloric load in the stomach with its complex digestive effects

- the inhibition of hepatic OATPs and P-gp which can decrease the entero-hepatic circulation of talinolol

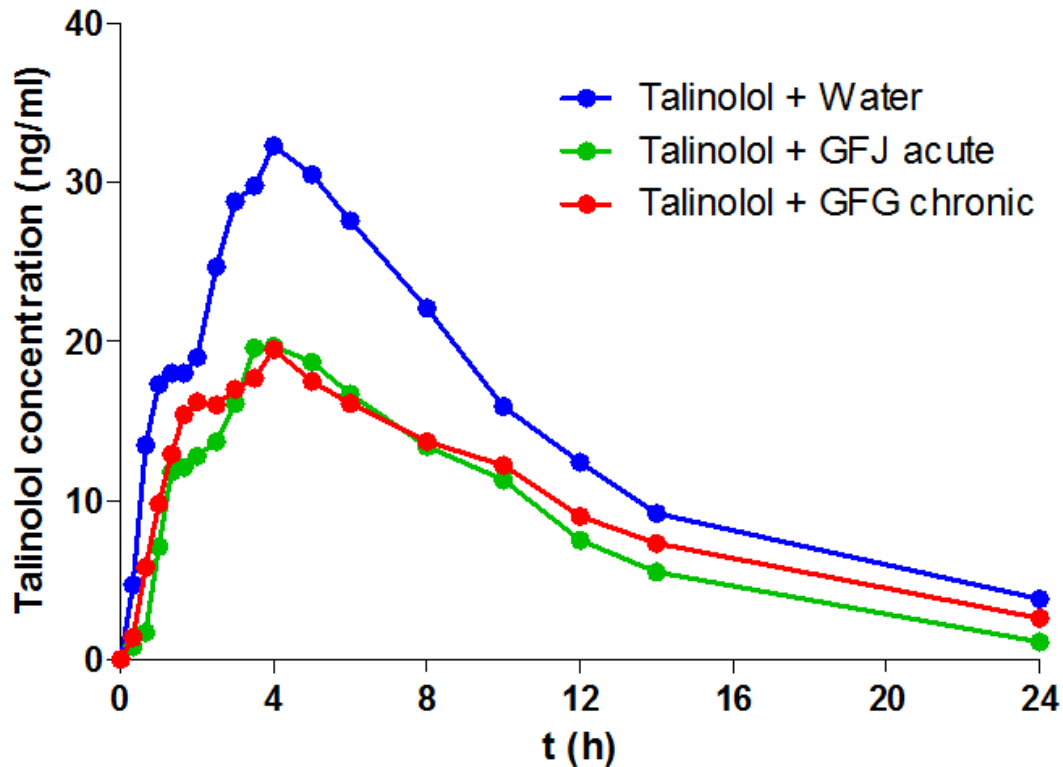


Figure 18: Pharmacokinetics of 50 mg talinolol ingested together with water, with 300 ml grapefruit juice (GFJ acute) or after 6 days of 300 ml daily grapefruit juice ingestion (GFJ chronic). Both acute and chronic ingestions of GFJ led to a decreased absorption for talinolol (modified according to Schwarz et al. 2005).⁶²

Rifampin-talinolol interaction:

Another example of interactions that can be seen more clearly in respect of *in-vitro* data is the interaction between talinolol and rifampin. The chronic treatment with rifampin, significantly decreased the areas under the curve of intravenous and oral talinolol (Figure 19).¹⁵ However, we believe it is a mistake to assume that this

effect resulted solely from the induction of P-gp expression. Rather, our data suggest that other factors may be involved such as:

- Inhibition of intestinal P-gp by presence of rifampin during talinolol absorption, as an acute effect of rifampin which may increase Talinolol Bioavailability.⁶³
- Induction of intestinal P-gp by the chronic pretreatment which lowers the Bioavailability of talinolol.⁶³
- Inhibition of intestinal OATP2B1 which may additionally decrease the absorption of talinolol.⁶⁴
- Inhibition of hepatic OATP2B1/1B1 which reduces the hepatic uptake and secretion of talinolol and leads to a higher bioavailability.⁶⁴

The overall change in bioavailability is the net effect of all confounders.

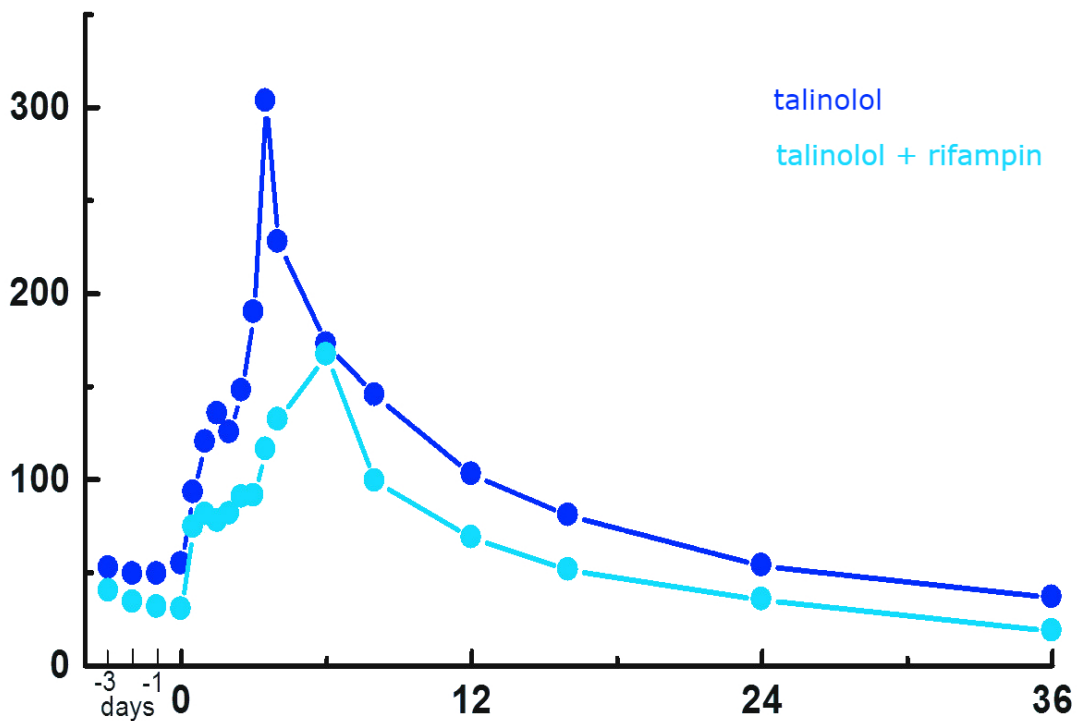
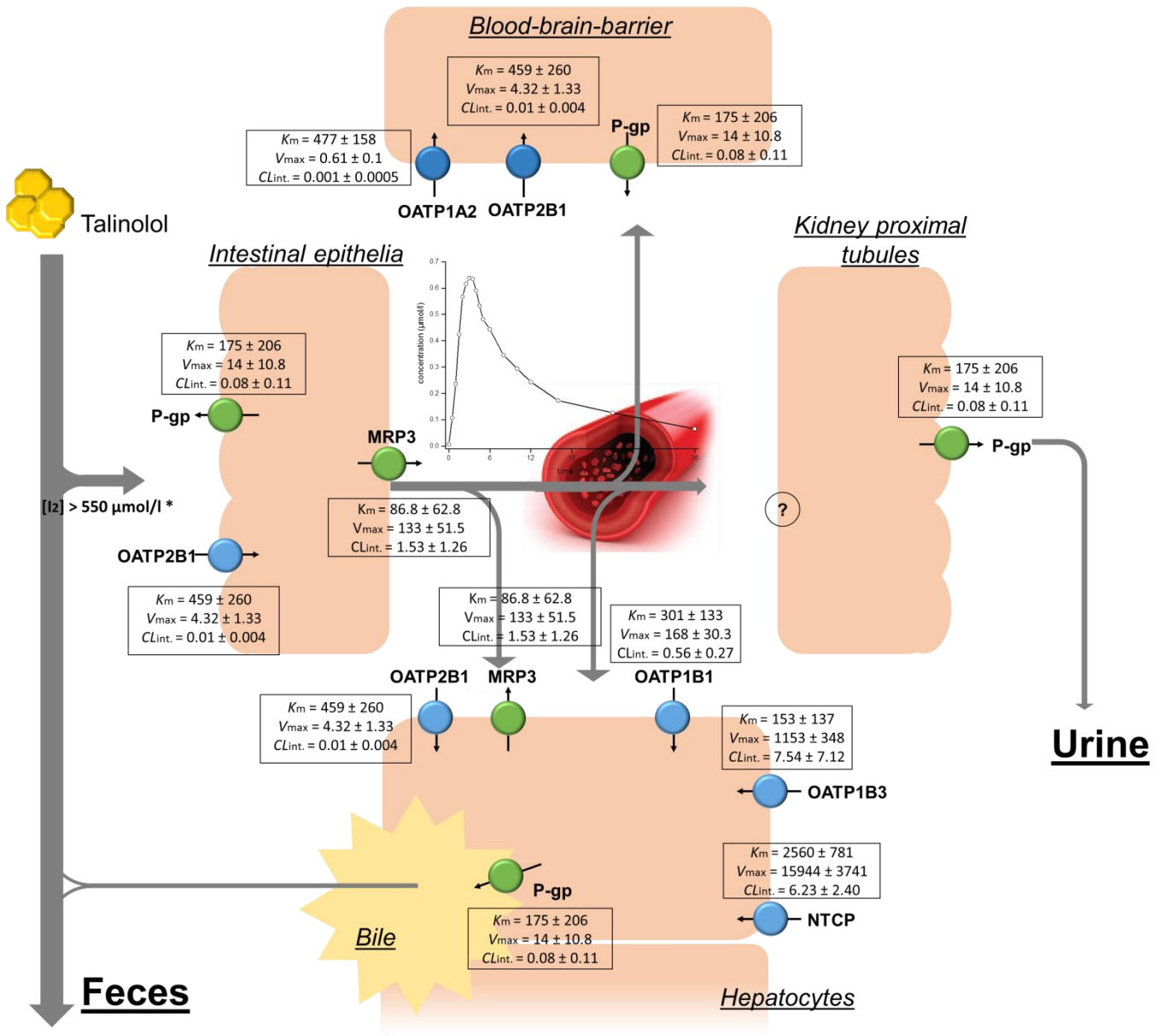


Figure 19: Pharmacokinetics of 100 mg talinolol (administered orally for 7 days) before and after the co-administration of rifampin (600 mg per day for 9 days). modified according to Westphal et al. 2000.¹⁵

In summary of all (Figure 20), talinolol is a selective β 1-adrenoceptor antagonist. It is highly soluble in water and has a pH-dependent partition coefficient, which puts it in the class III (high solubility, low permeability) of the BCS classification system.^{17,18} The drug is absorbed from the gastrointestinal tract in a mechanism which, according to our *in-vitro* data, results from a vectorial transport mediated by OATP2B1 and MRP3. However, talinolol absorption is erratic with an absolute bioavailability of 55–70%, and because talinolol undergoes nearly no metabolism^{12,13,15,19}, this low bioavailability could be better explained by P-gp efflux transporters, which lower its intestinal absorption. Talinolol is also known to be widely distributed ($V_d = 3\text{--}6$ l/kg) and to have an entero-hepatic circulation for about 10% of its intravenously administered dose.^{12,13,15,19,22,23} This can be also explained by our data, as talinolol showed to be transported by a number of drug transporters including the hepatic uptake transporters OATP1B1, OATP1B3 and OATP2B1. We have therefore concluded that the interplay among these uptake transporters and the hepatic efflux transporters P-gp and MRP3 defines the fraction of talinolol excreted to the bile. Regarding its excretion, talinolol undergoes an intensive intestinal secretion and a renal excretion by filtration and active secretion^{12,13,15,19,23}, which can be explained by the efflux transporter P-gp. However, the mechanism in which talinolol is transported from the blood stream into the enterocytes and the renal cells are still to be clarified.

Furthermore, this research is, to our knowledge, the first to demonstrate the vectorial transport of drugs *in-vivo*. This vectorial transport was previously demonstrated for the antihistamine agent fexofenadine using Caco-2 cells and was shown to involve specific apical uptake (OATP2B1) and efflux (p-gp) transporters as well as basolateral efflux transporters (MRP3).⁶⁵ Similarly, our retrospective pharmacogenomic data showed the absorption of talinolol to be significantly influenced by genetic variants of MRP3 and P-gp, whereas the highest absorption rate was seen in volunteers with deficient P-gp and normal MRP3 and the lowest in those with deficient MRP3 and normal P-gp.



K_m : $\mu\text{mol/l}$, V_{max} : $\mu\text{mol/mg}\times\text{min}$, $CL_{int.}$: $\text{ml}/\mu\text{g}\times\text{min}$

* considering a dose of 50mg talinolol, administered orally with 240 ml water

Figure 20: Transporters in plasma membrane domains of intestinal epithelia, hepatocytes, kidney proximal tubules and brain capillary endothelial cells and their potential role in talinolol pharmacokinetics.

7. Summary:

Introduction: The β 1-adrenergic receptor antagonist talinolol is a probe drug for P-glycoprotein (P-gp). It is absorbed erratically and incompletely from the gastrointestinal tract. However, its pharmacokinetics might also be influenced by further uptake and efflux transporters as concluded from interaction studies with naringin and verapamil in human. Additionally, the transcellular transport through the different tissues, including enterocytes, hepatocytes and kidney tubular cells, is not completely understood so far. Therefore, we aimed to measure the affinity of talinolol to drug transporting proteins (OCT1-3, PEPT1, OCTN2, ASBT, NTCP, MRP 1-3 and P-gp as well as OATP 1B1, 1B3, 2B1 and 1A2) and some of their genetic variants known to be of pharmacokinetic relevance (OATP1A2 *2 and *3 as well as OATP2B1 V201M, R312Q and S486F). In a further step, we retrospectively evaluated the impact of clinically relevant genetic polymorphisms of transporters on the pharmacokinetics of talinolol in healthy subjects.

Materials and Methods: Time and concentration-dependent uptake assays with [3 H]-talinalol were performed either in stable transfected HEK293 or MDCKII cells expressing OATP1A2 *1, *2 and *3, OATP1B1, OATP1B3, OATP2B1 (and its genetic variants p.V201M, p.R312Q and p.S486F), NTCP, ASBT, PEPT1, OCTN2, OCT 1-3 and the respective vector control or in inside-out lipovesicles expressing the efflux transporters MRP1-3 and P-gp. Talinalol was quantified by liquid scintillation counting. The transport rates were then corrected by the transporter proteomics measured in the cellular membrane. Regarding the pharmacogenomic evaluation, it was carried out retrospectively in 39 healthy subjects who had participated in former pharmacokinetic studies with talinalol. This evaluation included a variety of transporter related genetic variants, known to be of a clinical meaning for their substrates.

Results: Among the uptake transporters, talinalol was shown to be a substrate of OATP1B3 ($K_m = 153 \pm 137 \mu\text{mol/l}$; $V_{max} = 168 \pm 30.3 \mu\text{mol/mgxmin}$), OATP1B1 ($K_m = 301 \pm 133 \mu\text{mol/l}$; $V_{max} = 1135 \pm 348 \mu\text{mol/mgxmin}$), OATP2B1 ($K_m = 459 \pm 260 \mu\text{mol/l}$; $V_{max} = 4.32 \pm 1.33 \mu\text{mol/mgxmin}$), OATP1A2 ($K_m = 477 \pm 158 \mu\text{mol/l}$; $V_{max} = 0.61 \pm 0.1 \mu\text{mol/mgxmin}$) and NTCP ($K_m = 2560 \pm 781 \mu\text{mol/l}$; $V_{max} = 15944 \pm 3741 \mu\text{mol/mgxmin}$)

but not a substrate of OCT1-3, OCTN2, PEPT1 or ASBT. When it comes to the efflux transporters, talinolol was transported by both P-gp ($K_m = 175 \pm 206 \text{ mol/l}$; $V_{max} = 14 \pm 10.8 \text{ nmol/mgxmin}$) and MRP3 ($K_m = 86.8 \pm 62.8 \text{ } \mu\text{mol/l}$; $V_{max} = 133 \pm 51.5 \text{ } \mu\text{mol/mgxmin}$) but not by MRP2. The pharmacogenomic analysis supported the *in-vitro* results, as it showed a significant decrease in talinolol absorption (AUC and C_{max}) in subjects with the loss of function variant MRP3 211C>T and in those with a decreased P-gp function due to having less than 5 T-allels in the haplotype P-gp 1236-2677-3435-TTT. No significant changes were found associated with other transporters' genetic variants.

Conclusion: Our in-vitro results suggested the vectorial transport of talinolol through the enterocytes to consist mainly of apical OATP2B1 and P-gp and basolateral MRP3. Additionally in the hepatocytes, apical OATP1B1, OATP1B3 and NTCP seem to be involved as well. This vectorial transport was demonstrated in-vivo for the first time by our pharmacogenomic analysis, where talinolol absorption was significantly influenced by both P-gp and MRP3 genetic variants.

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9. Appendix:

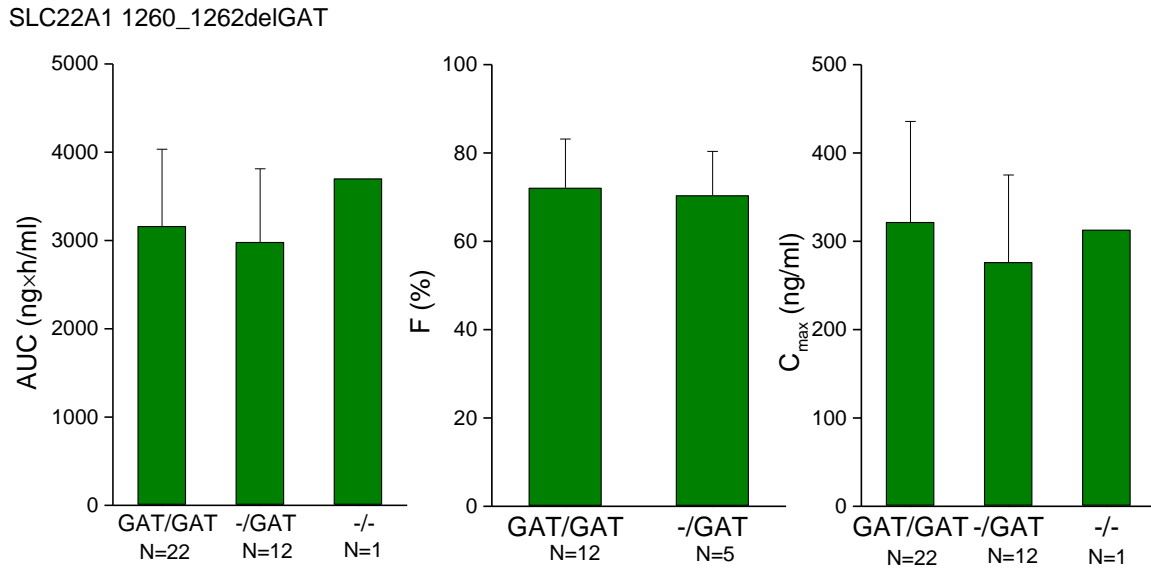


Figure S1: AUC, F and C_{max} of talinolol after single dose oral administration 100 mg talinolol in respect of the *SLC22A1* 1260_1262delGAT polymorphism.

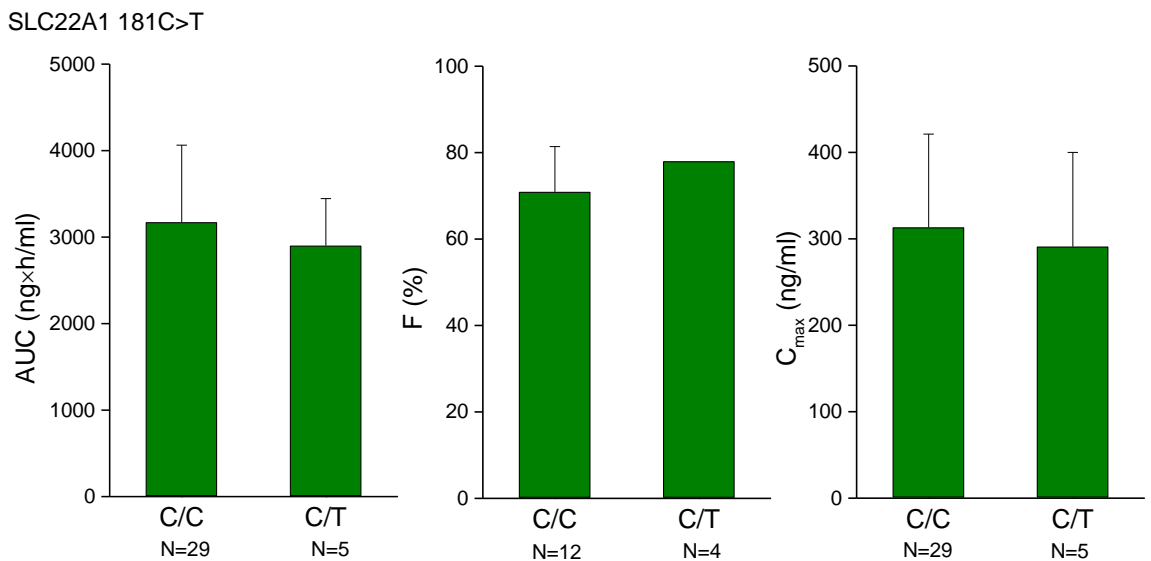


Figure S2: AUC, F and C_{max} of talinolol after single dose oral administration 100 mg talinolol in respect of the *SLC22A1* 181C>T polymorphism.

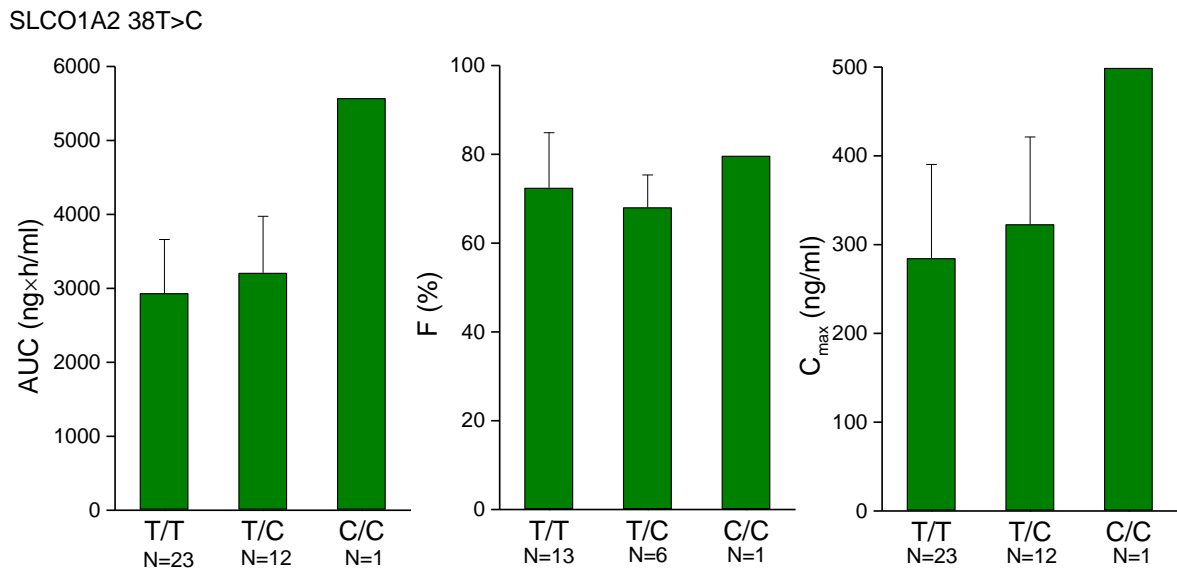


Figure S3: AUC, F and C_{max} of talinolol after single dose oral administration 100 mg talinolol in respect of the *SLCO1A2* 38T>C polymorphism.

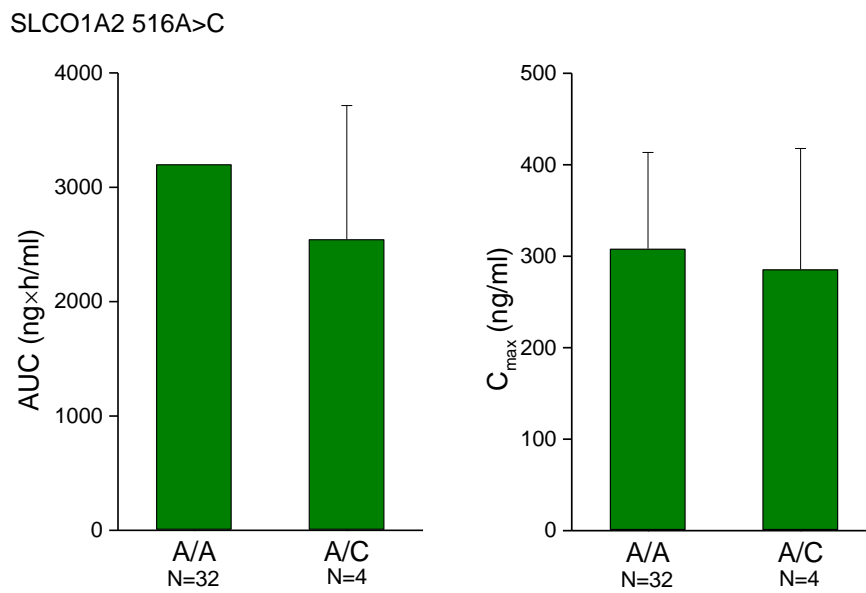


Figure S4: AUC and C_{max} of talinolol after single dose oral administration 100 mg talinolol in respect of the *SLCO1A2* 516A>C polymorphism.

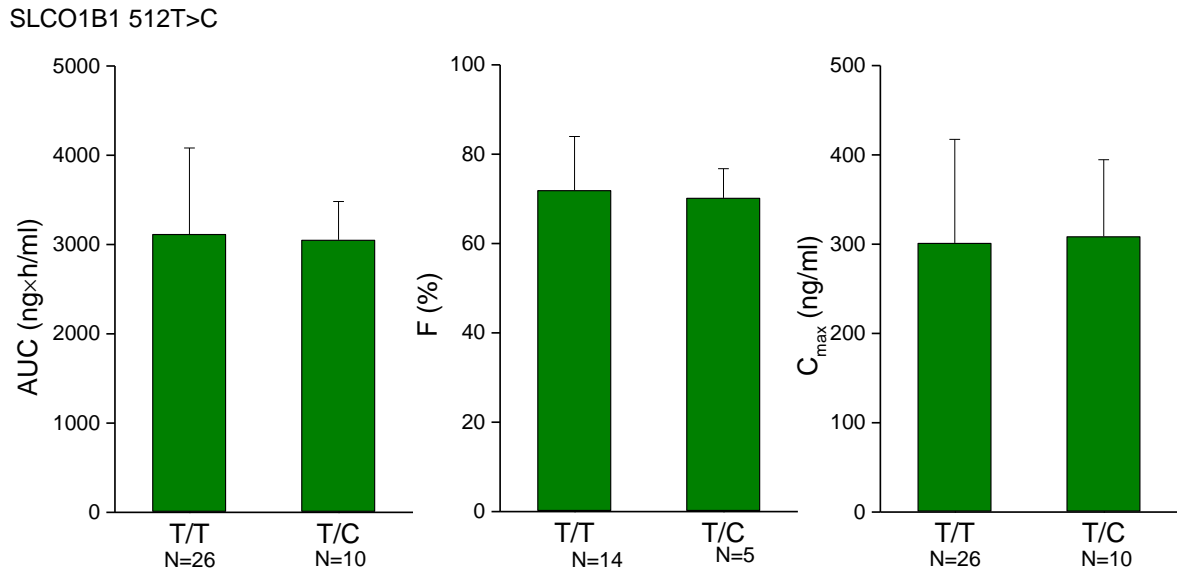


Figure S5: AUC, F and C_{max} of talinolol after single dose oral administration 100 mg talinolol in respect of the *SLCO1B1* 521T>C polymorphism.

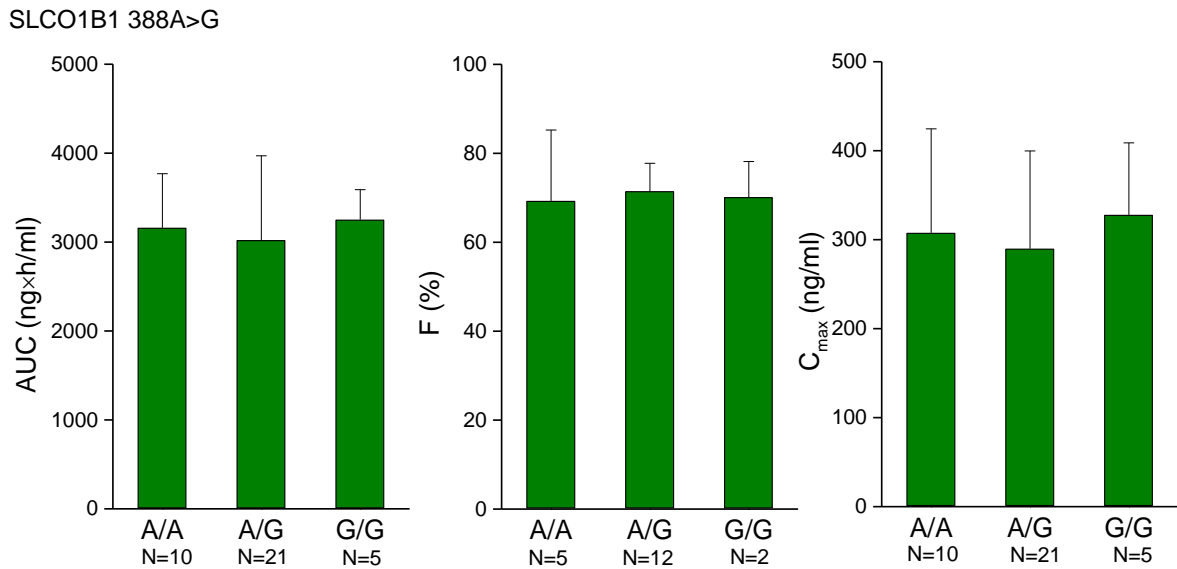


Figure S6: AUC, F and C_{max} of talinolol after single dose oral administration 100 mg talinolol in respect of the *SLCO1B1* 388A>G polymorphism.

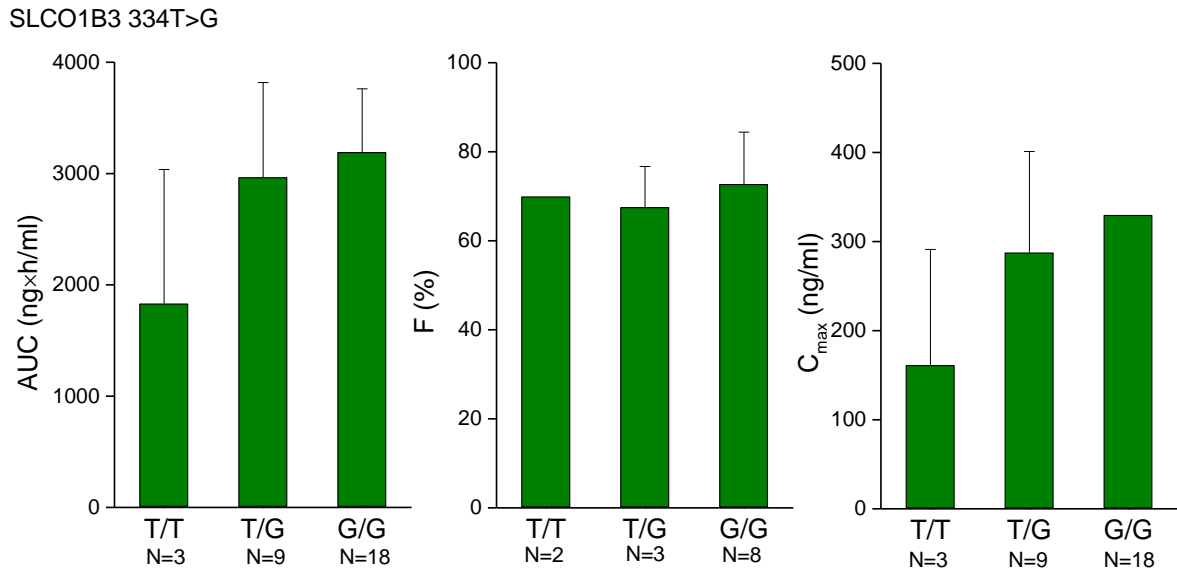


Figure S7: AUC, F and C_{max} of talinolol after single dose oral administration 100 mg talinolol in respect of the *SLCO1B3* 334T>G polymorphism.

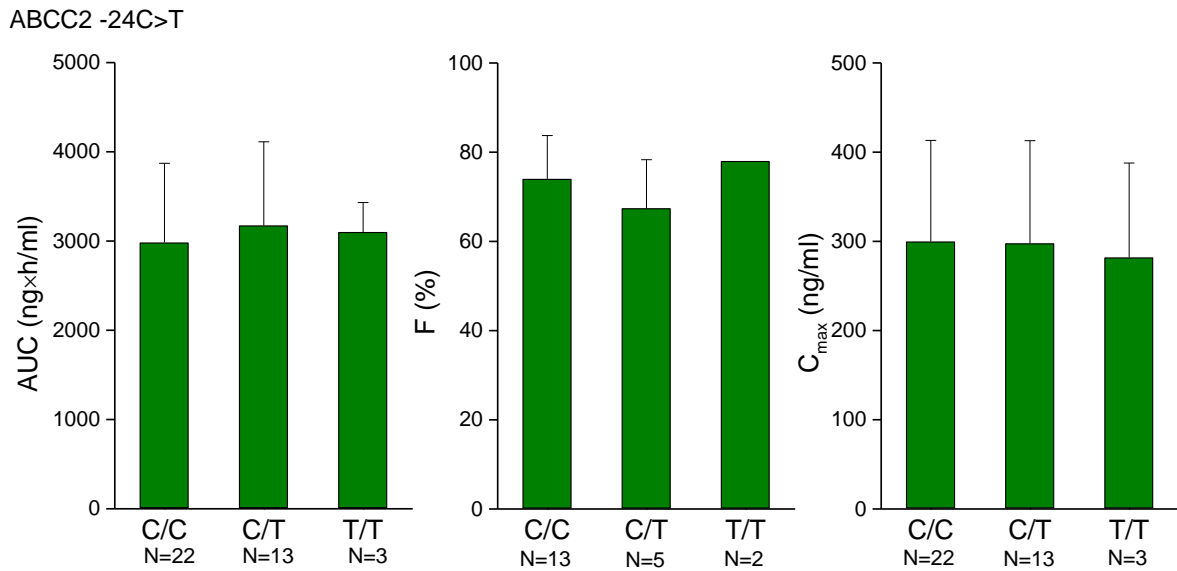


Figure S8: AUC, F and C_{max} of talinolol after single dose oral administration 100 mg talinolol in respect of the *ABCC2* -24C>T polymorphism.

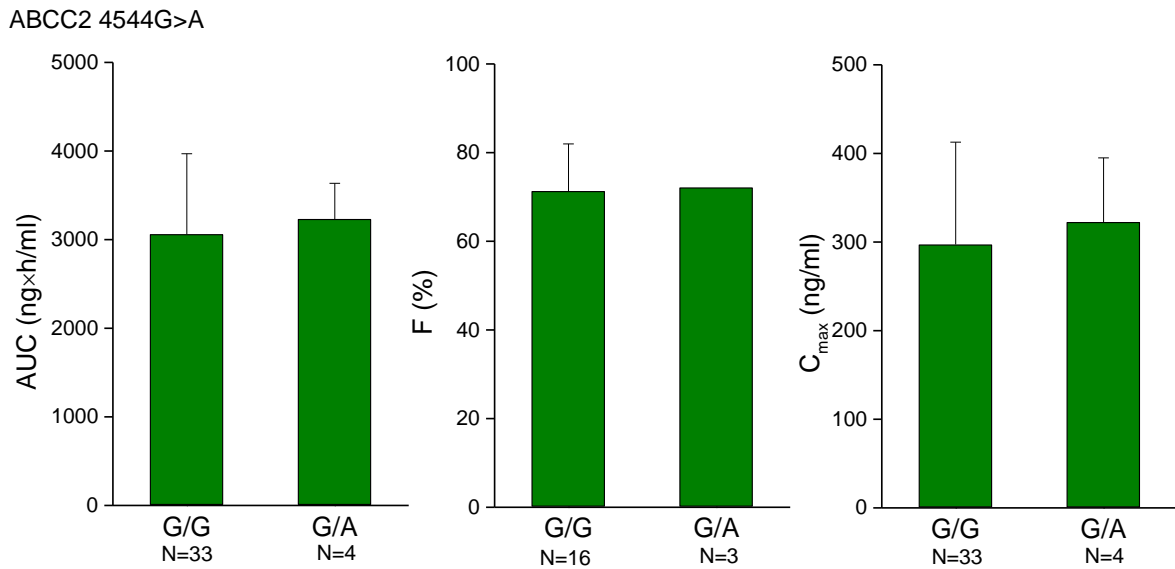


Figure S9: AUC, F and C_{max} of talinolol after single dose oral administration 100 mg talinolol in respect of the *ABCC2 4544G>A* polymorphism.

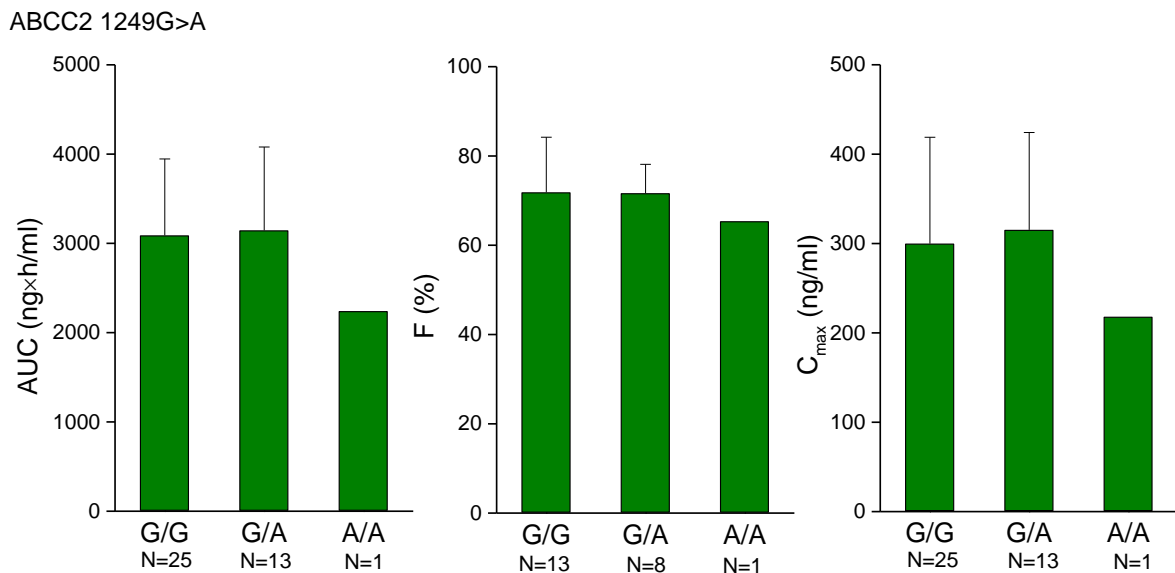


Figure S10: AUC, F and C_{max} of talinolol after single dose oral administration 100 mg talinolol in respect of the *ABCC2 1249G>A* polymorphism.

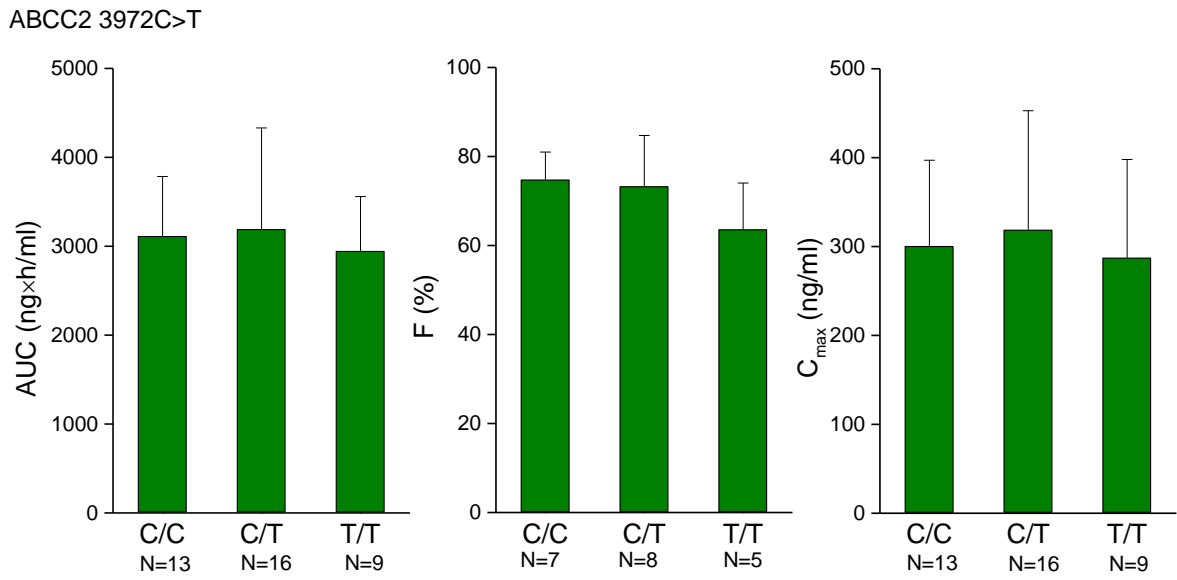


Figure S11: AUC, F and C_{max} of talinolol after single dose oral administration 100 mg talinolol in respect of the *ABCC2* 3972C>T polymorphism.

Table S1: Pharmacokinetic characteristics (mean \pm SD) of talinolol after single dose oral administration of 100 mg talinolol in respect of the genetic variants of OCT1, OATP1A2, OATP1B1, OATP1B3 and MRP2.

<i>Genetic variant</i>	genotype	AUC (ngxh/ml)	F (%)	C _{max} (ng/ml)	T _{1/2} (h)	CL _{ren} (ml/min)
<i>SLC22A1</i> <i>1260_1262</i> <i>delGAT</i>	GAT/GAT	3164 \pm 870	72 \pm 11	322 \pm 114	13 \pm 1.9	193 \pm 55
	-/GAT	2983 \pm 830	70 \pm 10	277 \pm 99	12.5 \pm 1.5	191 \pm 71
	-/-	3703	-	313	15.8	-
<i>SLC22A1</i> <i>181C>T</i>	C/C	3172 \pm 891	71 \pm 11	313 \pm 108	12.8 \pm 1.9	197 \pm 69
	C/T	2902 \pm 544	78	291 \pm 109	13 \pm 1.9	211 \pm 30
<i>SLCO1A2</i> <i>38T>C</i>	T/T	2935 \pm 726	73 \pm 12	285 \pm 106	12.8 \pm 1.9	205 \pm 61
	T/C	3209 \pm 766	68 \pm 7	323 \pm 98	13 \pm 1.8	205 \pm 69
	C/C	5572	80	499	13	92
<i>SLCO1A2</i> <i>516A>C</i>	A/A	3202 \pm 769	71 \pm 10	308 \pm 105	12.9 \pm 1.9	197 \pm 66
	A/C	2546 \pm 1168	-	286 \pm 132	13 \pm 0.8	208
<i>SLCO1B1</i> <i>512T>C</i>	T/T	3117 \pm 964	72 \pm 12	301 \pm 116	12.9 \pm 1.9	190 \pm 57
	T/C	3054 \pm 429	70 \pm 7	309 \pm 86	12.6 \pm 1.4	230 \pm 85
<i>SLCO1B1</i> <i>388A>G</i>	A/A	3161 \pm 609	69 \pm 16	308 \pm 117	13.9 \pm 1.8	173 \pm 46
	A/G	3023 \pm 947	72 \pm 6	290 \pm 110	13.3 \pm 3.2	221 \pm 73
	G/G	3252 \pm 339	70 \pm 8	328 \pm 81	12 \pm 1.3	176 \pm 61
<i>SLCO1B3</i> <i>334T>G</i>	T/T	1832 \pm 1204	70	161 \pm 130	17.4 \pm 6.5	181 \pm 39
	T/G	2967 \pm 850	68 \pm 9	288 \pm 113	12.6 \pm 2.4	235 \pm 64
	G/G	3192 \pm 569	73 \pm 12	330 \pm 101	12.5 \pm 1.7	191 \pm 60
<i>ABCC2</i> <i>1249G>A</i>	G/G	3090 \pm 856	72 \pm 12	300 \pm 119	13.1 \pm 3	198 \pm 65
	G/A	3145 \pm 935	72 \pm 7	315 \pm 109	13.4 \pm 1.8	196 \pm 73
	A/A	2240	65	218	13.2	209
<i>ABCC2</i> <i>4544G>A</i>	G/G	3061 \pm 908	71 \pm 11	297 \pm 116	13 \pm 2.8	195 \pm 65
	G/A	3233 \pm 403	72	323 \pm 73	12.6 \pm 1.2	268
<i>ABCC2</i> <i>3972C>T</i>	C/C	3116 \pm 668	75 \pm 6	301 \pm 96	13.3 \pm 3.8	230 \pm 73
	C/T	3193 \pm 1134	73 \pm 11	319 \pm 134	13 \pm 1.8	186 \pm 68
	T/T	2947 \pm 612	64 \pm 10	288 \pm 110	13.7 \pm 1.8	178 \pm 38
<i>ABCC2</i> - <i>24C>T</i>	C/C	2983 \pm 889	74 \pm 10	300 \pm 113	13 \pm 3	214 \pm 59
	C/T	3175 \pm 937	67 \pm 11	298 \pm 115	13.3 \pm 1.8	171 \pm 74
	T/T	3101 \pm 330	78	282 \pm 106	14.1 \pm 3.3	208 \pm 35