Pharmacogenetic Determinants of Atorvastatin Metabolism and Response

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Diplom-Biologe (technisch orientiert)

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aus Memmingen

Tübingen

2011

Tag der mündlichen Qualifikation:	05.08.2011
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For my parents, Annemarie and Alois,

and for my soon to be wife, Arian.

Abbreviations

ABCG2, ATP-binding cassette transporter sub-family G member 2
ATV, atorvastatin
AUC, area under the curve
bp, base pair
BSA, bovine serum albumin
cDNA, complementary DNA
CES, carboxylesterase
CYP, cytochrome P450
Da, dalton (unified atomic mass unit)
DCA, deoxycholic acid
DMEM, Dulbecco's modified eagle medium
DNA, deoxyribonucleotide triphosphate
DTT, dithiothreitol
EDTA, ethylendiamintetraacetic acid
HDL, high density lipoprotein
HMG Co A, 3-hydroxy 3-methylglutaryl coenzyme A
HR, hazard ratio
HPLC, high-performance liquid chromatography
K_m, Michaelis-Menten constant
LCA, lithocholic acid
LDL, low density lipoprotein
MALDI TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MEM, minimum essential medium
mRNA, messenger ribonucleic acid
NADPH, nicotinamide adenine dinucleotide phosphate
OATP1B1, organic anion transporting polypeptide, family, 1 sub-family B, member 1
o-OH, orthohydroxy
OR, odds ratio
PAGE, polyacrylamide gel electrophoresis
PCR, polymerase chain reaction
p-OH, parahydroxy
PON, paraoxonase
RNA, ribonucleic acid
r_s, Spearman coefficient
SAP, shrimp alkaline phosphatase
SDS, sodiumdodecylsulfate
SNP, single nucleotide polymorphism
TAE, tris-acetate-EDTA
TBS, tris buffered saline
UDP, uridine 5'-diphosphate
UDPGA, uridine 5'-diphospho-glucuronic acid
UGT, uridine 5'-diphosphate glucuronosyltransferases
UTR, untranslated region
VAF, variant allele frequency
V_{max}, maximum reaction rate

Zusammenfassung

Erhöhte LDL-Cholesterinwerte sind eindeutig mit einem gesteigerten Risiko für Arteriosklerose und koronare Herzerkrankungen assoziiert. Diese Herzerkrankungen stellen die Haupttodesursache in entwickelten Ländern dar und ihre Zahl steigt rapide in Schwellen- und Entwicklungsländern. Statine, zum Beispiel das im Fokus dieser Dissertation stehende Atorvastatin, sind potente HMG-CoA Reduktase Inhibitoren, die den LDL-Cholesterinwert und damit das Risiko für koronare Herzerkrankungen effektiv senken können. Obwohl Statine generell gut vertragen werden, sind unerwünschte Arzneimittelwirkungen wie Myopathie, Rhabdomyolyse und Hepatotoxizität gut belegt. Neue Erkenntnisse deuten auf eine mögliche Rolle bestimmter Atorvastatin-Metabolite bei der Entstehung von Myopathien hin. Beispielsweise zeigten Patienten, die unter Atorvastatin-Behandlung an Myopathie litten, erhöhte Plasmawerte des pharmakologisch inaktiven Metaboliten Atorvastatin-Lakton. Zusätzlich hatte eine *in vitro* Studie eine erhöhte Toxizität dieses Metaboliten im Vergleich mit dem Medikament selbst, der Atorvastatin-Säure, gezeigt.

Ziel dieser Arbeit war es, den Metabolismus von Atorvastatin unter besonderer Berücksichtigung von Atorvastatin-Lakton zu untersuchen. Dazu sollten unter Verwendung einer Kohorte von Leberproben die beteiligten Enzyme, sowie einflussreiche genetische und nicht-genetische Faktoren der Enzymreaktionen identifiziert werden. Zuerst konzentrierte sich diese Arbeit auf die Identifizierung des Enzyms, das für die Bildung von Atorvastatin-Lakton verantwortlich ist. Messungen mit 9 rekombinant erzeugten Isoformen von UDP-Glukuronosyltransferasen der Gruppen 1A und 2B und kinetische Daten von rekominantem UGT1A1 und UGT1A3 deuteten auf eine wichtige Rolle der letztgenannten Enzyme bei der Laktonisierung von Atorvastatin hin. Zusätzlich besteht am Dr. Margarete Fischer-Bosch Institut für klinische Pharmakologie eine Sammlung humaner Lebern von kaukasischen Spendern mit weitreichender klinischer Dokumentation sowie geno-und phänotypischer Charakterisierung. Quantitative Analysen der Protein- und mRNA-Expression von UGT1A1 und UGT1A3 wurden in dieser Sammlung durchgeführt. Anschließende Korrelationsanalysen mit ebenfalls durchgeführten Messungen zur Bildung von Atorvastatin-Lakton in humanen Lebermikrosomen dieser Kohorte identifizierten eindeutig UGT1A3 als wichtigstes Enzym zur Bildung von Atorvastatin-Lakton. Darüberhinaus konnten eine hohe Variabilität der Lakton-Bildung, sowie der Expressions-Level von UGT1A3 beobachtet werden.
Daher wurde im *UGT1A3*-Gen nach Polymorphismen gesucht, die die Bildung von Atorvastatin-Lakton signifikant beeinflussen und möglicherweise die beobachtete Variabilität

erklären. In der Tat konnte das *UGT1A3*2* Allel mit einer signifikant erhöhten Expression von UGT1A3 mRNA und Protein als auch mit einer signifikant erhöhten Bildung von Atorvastatin-Lakton *in vitro* wie oben beschrieben, als auch *in vivo* in einer darauffolgenden Studie, in Zusammenhang gebracht werden. Diese retrospektive, pharmakogenetische Analyse von Proben gesunder Probanden, die eine Einzeldosis Atorvastatin erhalten hatten, konnte die *in vitro* Ergebnisse untermauern, da in *UGT1A3*2* Trägern *in vivo* ein signifikant höheres AUC-Verhältnis von Atorvastatin-Lakton zur Säure gefunden wurde. Weitere *in vitro* Untersuchungen konnten darüber hinaus auch eine signifikant höhere Glukuronidierung anderer UGT1A3-Substrate (Estron, Deoxichol- (DCA), und Lithocholsäure (LCA)) zeigen. Experimente mit rekombinant erzeugtem *UGT1A3*1* und *UGT1A3*2* wiesen eine etwa 8-fach höhere Expression und eine etwa 5- bis 6-fach niedrigere spezifische Aktivität (DCA und LCA) von *UGT1A3*2* auf. Die Bildung von Atorvastatin-Lakton in *UGT1A3*2* homozygoten Proben lag im Mittel etwa 2-fach höher als im Wildtyp. Zusammengefasst zeigen diese Daten, dass die Aminosäure-Austausche in *UGT1A3*2* (W11R, V47A) mit einer niedrigeren spezifischen Aktivität des Enzyms, aber auch einer erhöhten Expression von *UGT1A3* assoziiert sind. Diese erhöhte Expression kann jedoch nicht durch die untersuchten Promotor-SNPs in *UGT1A3*2* (*-758A>G, -751T>C, -581C>T, -204A>G* und *-66T>C*) erklärt werden.

Im nächsten Schritt lag der Fokus der Untersuchung auf Seite der Hydrolyse von Atorvastatin-Lakton, einer Reaktion, die die Konzentration dieses Metaboliten ebenfalls beeinflussen sollte. Aus der Leber-Sammlung standen genomweite mRNA Expressiondaten zur Verfügung, die eine Korrelation mit zuvor ermittelten Werten der *in vitro* Hydrolyse von Atorvastatin-Lakton zurück zur Säure möglich machten. Von so gefundenen Kandidaten wurde die mRNA- und Proteinmenge in den Leberproben quantifiziert und ebenfalls mit den Daten zur Hydrolyse korreliert. Somit konnten PON1 und PON3 als verantwortliche Enzyme dieser Reaktion identifiziert werden. Die anschließende intensive Genotypisierung des PON-Locus ermöglichte Assoziationsstudien mit den Werten zur Hydrolyse von Atorvastatin-lakton. Somit war es möglich zu zeigen, dass der Haplotyp *PON*1* mit signifikant niedrigerer PON1 mRNA- und Proteinexpression als auch mit signifikant niedrigerer Hydrolyse von Atorvastatin-Lakton assoziiert ist. Insbesondere wurde nachgewiesen, dass Polymorphismen im Promotor von PON1 (*-108 T>C, -832 G>A, -1741 G>A*) sowie eine stark genetisch gekoppelte Gruppe von PON3 Polymorphismen (*-4984 A>G, -4105 G>A, -1091 A>G, -746 C>T* and F21; rs13226149) mit diesen beobachteten Unterschieden in Verbindung stehen. Trotz eingehender Genotypisierung von *PON3* wurde jedoch kein häufiger Polymorphismus

mit Einfluss auf die PON3 Expression gefunden. Jedoch wurde ein relativ seltener Polymorphismus im Bereich zwischen *PON3* und *PON1* identifiziert, der mit signifikant erhöhtem PON3 assoziiert ist und so möglicherweise eine erhöhte Hydrolyse von Atorvastatin-Lakton anzeigt.

Schließlich wurde in dieser Arbeit auch untersucht, ob die gefundene genetische Variabilität in *UGT1A3* und im *PON*-Locus, die *in vitro* signifikant die Bildung beziehungsweise Hydrolyse von Atorvastatin-Lakton beeinflusst hatte, auch *in vivo* das Risiko von unerwünschten Arzneimittelwirkungen oder das Ansprechen auf eine Therapie mit Atorvastatin verändert. Zu diesem Zweck wurden in einem Kooperationsprojekt 1180 Proben von Patienten der „Deutschen Diabetes Dialyse Studie“ retrospektiv auf ausgewählte Polymorphismen genotypisiert, die bei einem mittleren follow-up von 4 Jahren mit Atorvastatin oder Placebo behandelt worden waren. Ausgewählt wurden neben *UGT1A3* und *PON*-Locus Marker-Polymorphismen auch solche der Gene *ABCG2*, *CYP3A4* und *CYP3A5*, zu denen Literaturstellen einen möglichen Einfluss auf den Metabolismus von Atorvastatin gezeigt hatten. Zur Verfügung stehende Daten dieser Studie erlaubten eine Analyse zum Einfluss dieser Polymorphismen auf den Studienendpunkt (Tod durch kardiovaskuläre Ursachen, nicht-tödlicher Herzinfarkt, sowie Schlaganfall) und auf Lipid-Spiegel im Plasma (Triglycerid-, LDL- und HDL-Cholesterin-Spiegel). Eine Analyse zum direkten Einfluss auf die Atorvastatin-Lakton Spiegel war nicht möglich, da diese pharmakokinetischen Daten nicht vorhanden waren. Die untersuchten Polymorphismen zeigten, abhängig von der Atorvastatin-Therapie, keine signifikanten Unterschiede zwischen Trägern und Nicht-Trägern bezüglich des Studienendpunktes oder der Lipid-Spiegel. Interessant war jedoch, dass signifikant weniger Endpunkte in *UGT1A3*2* Homozygoten beider Gruppen auftraten. Eine Erklärung hierfür könnte die starke genetische Kopplung dieser Polymorphismen mit der *UGT1A1*28* Variante sein. Diese führt zu niedrigerer UGT1A1 Expression, somit zum beobachteten signifikant erhöhten Spiegel des potenten Antioxidans Bilirubin und damit wohl zu einer signifikant gesteigerten Überlebensrate in dieser Patientenpopulation.

Da diese Studie nicht für diesen Zweck entworfen wurde, sollten aus zwei Gründen weitere Untersuchungen durchgeführt werden: Erstens war es nicht möglich, einen Einfluss auf die Atorvastatin-Lakton Spiegel zu untersuchen, da diese Daten nicht zur Verfügung standen. Zweitens besteht die Möglichkeit, dass die spezifischen Charakteristika dieser Studie mit Diabetes-Patienten auf Dialysebehandlung die Ergebnisse beeinflusste, da zum Beispiel

Plasma-Spiegel von Paraoxonasen in Diabetes Patienten signifikant niedriger sind, als in gesunden Probanden.

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass genetische Varianten in *UGT1A3* und im *PON*-Locus die Entstehung beziehungsweise Hydrolyse von Atorvastatin-Lakton, einem möglicherweise toxischeren Metaboliten, *in vitro* signifikant beeinflussen. *In vivo* war *UGT1A3*2* in der Tat mit einem signifikant höheren Atorvastatin-Lakton zu -Säure AUC Verhältnis in gesunden Probanden assoziiert, die eine Atorvastatin Einzeldosis erhalten hatten. Keine der untersuchten Varianten zeigte jedoch in einer Studie mit Diabetes-Patienten auf Dialysebehandlung einen signifikanten, Atorvastatin-abhängigen Effekt auf das Auftreten von Studien-Endpunkten oder von Lipid-Plasmaspiegeln.

Summary

High levels of LDL-cholesterol are associated with an increased risk for atherosclerosis and cardiovascular events. Incidence rates of cardiovascular events are rising in developing countries and have reached the lead as cause of death in developed countries. Statins, like atorvastatin, are very effective HMG-CoA reductase inhibitors, successfully lowering LDL-cholesterol and the risk for cardiovascular events. They are tolerated well by the majority of patients but low efficacy and adverse events, like myopathy, rhabdomyolysis, or hepatotoxicity, are well documented. Recent reports point to an involvement of certain metabolites of atorvastatin in the development of myopathy. Atorvastatin-lactone was increased in patients suffering from atorvastatin-induced myopathy and this pharmacologically inactive metabolite had stronger cytotoxic effects on primary human muscle cells.

Therefore this study focused on the identification of genetic variability, which might increase the plasma-level of atorvastatin-lactone. First, this work focused on the identification of the enzyme responsible for the formation of atorvastatin-lactone. Incubations with 9 recombinant isoforms of UDP-glucuronosyltransferases (UGTs) of the groups 1A and 2B and kinetic data of recombinant enzymes UGT1A1 and UGT1A3 pointed to an important role of these enzymes in the lactonization of atorvastatin. A collection of Caucasian human liver samples with extensive clinical documentation, as well as, genotypic and phenotypic characterization is available at the Dr. Margarete Fischer-Bosch institute of clinical pharmacology. Correlation analyses of quantitative protein and mRNA expression data of the candidate genes UGT1A1 and UGT1A3 with lactonization of atorvastatin in human liver microsomes generated from this collection identified UGT1A3 as the most important enzyme in atorvastatin lactone formation. Additionally, a high population-variability was observed for this activity, as well as, for UGT1A3 expression. Hence, *UGT1A3* was genotyped to investigate whether polymorphisms might explain this variability. The common *UGT1A3*2* allele was associated with significantly increased mRNA and protein expression of UGT1A3 and increased formation of atorvastatin-lactone. Retrospective genotyping of healthy volunteers having received a single dose of atorvastatin revealed a significantly increased AUC ratio of atorvastatin-lactone to -acid in *UGT1A3*2* carriers, which confirmed the *in vitro* observations of increased lactonization of this genotype. Further *in vitro* experiments showed also significantly increased glucuronidation of other UGT1A3 substrates (estrone, deoxycholic-(DCA) and lithocholic-acid (LCA)) in carriers of this allele. Experiments comparing

recombinant *UGT1A3*1* (wild-type) with *UGT1A3*2* showed that this allele increased the expression of the recombinant protein about 8-fold and decreased the specific activity approximately 5- to 6-fold (DCA and LCA). Interestingly, lactonization of atorvastatin in the Caucasian collection was increased 2-fold in homozygotes of *UGT1A3*2*, which might be explained by a compensatory effect that results from an increased expression level and a decreased specific activity of the **2* haplotype. In conclusion, these data show that the amino acid changes in *UGT1A3*2* (W11R, V47A) are associated with a decreased specific activity of the enzyme, but, on the other hand also with an increased expression level of *UGT1A3*. This increased expression cannot be explained by the analyzed promoter SNPs of *UGT1A3*2* (*-758A>G, -751T>C, -581C>T, -204A>G* und *-66T>C*).

In the second part, this work concentrated on the reverse process, the hydrolysis of atorvastatin-lactone, which also influences the levels of this metabolite. Genome-wide gene mRNA expression data available from the liver-collection allowed correlation analyses with the hydrolysis of atorvastatin-lactone and the identification of candidate genes for this reaction. Following this, quantitative protein and mRNA expression data of two candidates were generated and correlated with the hydrolysis of atorvastatin-lactone. In this way, PON1 and PON3 were identified as the enzymes most probably responsible for the hydrolysis of atorvastatin-lactone. Intensive genotyping within the *PON*-locus and association studies with the hydrolysis of atorvastatin-lactone were performed. The most common haplotype *PON*1* was found to be associated with significantly lower PON1 mRNA and protein expression, as well as, significantly lower hydrolysis of atorvastatin-lactone. *PON1* promoter polymorphisms *-108 T>C, -832 G>A, -1741 G>A* and a tightly linked group of PON3 polymorphisms (*-4984 A>G, -4105 G>A, -1091 A>G, -746 C>T* and F21; rs13226149) were associated with these changes. Although the genotyping approach focused mainly on *PON3* polymorphisms, no common influential *PON3* variations were identified and only one rare intergenic variation was found, which significantly increased PON3 protein expression and which (non-significantly) increased hydrolysis of atorvastatin-lactone. It was analyzed with cooperation partners whether the identified variability in *UGT1A3* and the *PON*-locus, which *in vitro* significantly changed atorvastatin-lactone levels, also changed the *in vivo* risk for adverse events or the response to atorvastatin-treatment in a large patient cohort. Samples from 1180 diabetes patients on dialysis from “Die deutsche Diabetes Dialyse Studie”, which had been either treated with atorvastatin or placebo and followed up for a mean of 4 years, were retrospectively genotyped for selected marker polymorphisms. The analysis included

UGT1A3 and *PON*-locus marker polymorphisms, as well as, polymorphisms of *ABCG2*, *CYP3A4* and *CYP3A5*, of which literature data made an influence on atorvastatin treatment outcome plausible. Data from this study allowed the analysis of the influences of these polymorphisms on study endpoint (death from cardiac causes, nonfatal myocardial infarction and stroke) and on lipid levels (triglycerides, LDL- and HDL-cholesterol). However, an analysis of the direct effect on atorvastatin-lactone levels was not detectable as no pharmacokinetic data were available. None of the analyzed polymorphisms showed significant differences between carriers and non-carriers on the study endpoint or on lipid levels of this patient cohort. An interesting observation was the significantly decreased number of endpoints in *UGT1A3*2* homozygotes in the atorvastatin and in the placebo receiving group. This might be explained by high linkage of this variant with *UGT1A1*28*, resulting in decreased UGT1A1 expression leading to significantly increased plasma levels of the potent antioxidant bilirubin and in this way, possibly, to significantly prolonged survival of this group, as previously described.

Because the study was not designed for this purpose, additional studies should be carried out in the future, mainly because: first, the effect on atorvastatin-lactone levels could not be analyzed, as these data were not available in the study, and second, the characteristics of this cohort of diabetes patients on dialysis might bias the results, as for example, the plasma levels of paraoxonases are known to be significantly decreased in diabetes patients.

In summary, this work characterized genotype-phenotype associations at the *UGT1A3* and the *PON1/PON3*-loci in a large human liver-bank. *In vitro*, the UGT1A3*2 haplotype and several *PON*-locus polymorphisms were identified as factors associated with significantly increased levels of atorvastatin-lactone, a metabolite, which has been associated with toxicity. *In vivo*, the UGT1A3*2 allele was associated with a significantly higher atorvastatin-lactone to -acid AUC in a study with healthy volunteers, but none of the analyzed variants had a significant atorvastatin-dependent effect on survival or lipid levels in a study with diabetes patients on dialysis.

1. Introduction

1.1 Arteriosclerosis and the role of oxidized LDL

Along with the rising incidence of obesity and diabetes in developing countries, cardiovascular diseases are soon expected to be the main cause of death globally (Hansson, 2005). In most of the patients, atherosclerosis is the underlying disorder causing these types of diseases (Liao and Laufs, 2005). Atherosclerotic lesions, already observed by the German pathologist Virchow 100 years ago in patients dying of occlusive vascular disease (Tobert, 2003), are thickenings of the intima, the innermost layer of the artery and consist of cells, debris, connective tissue and lipids. Hypercholesterolemia, particularly increased levels of low-density lipoproteins (LDL), is the most important risk factor for the development of atherosclerotic lesions. Studies in animals and humans have shown that high levels of LDL-cholesterol are leading to increased retention of these lipids in the intima where it may be enzymatically and oxidatively modified. Modified LDL-cholesterol leads to the activation of inflammation processes, which is an important factor in plaque formation followed by years of intracellular and extracellular lipid accumulation in the intima. Rupture of atherosclerotic lesions occurs where the fibrous cap of atherosclerotic plaques is thin due to activated inflammatory processes, including the activation of proteolytic enzymes. Plaque ruptures expose prothrombic material from the atherosclerotic lesions and are the reason for 60 to 70 percent of coronary thromboses (Hansson, 2005).

1.2 Lowering LDL-cholesterol by statins

As the link between elevated plasma LDL-cholesterol and increased risk for cardiovascular diseases is clearly proven, a reduction of LDL-cholesterol is expected to be beneficial. Cholesterol homeostasis within the human body is maintained by endogenous synthesis in the liver, oral absorption from diet, excretion and resorption. Therefore, LDL-cholesterol-lowering strategies include dietary interventions, drugs to decrease cholesterol-uptake from the diet, sequestration from the resorption cycle of bile acids, which are important metabolites and a major route of cholesterol elimination from the body or, most efficiently, drugs to decrease the biosynthesis of cholesterol. The cholesterol biosynthesis is complex and involves more than 30 enzymes (Tobert, 2003). Statins, which block the HMG-CoA reductase, are the most effective drugs inhibiting the cholesterol biosynthesis. This blockade induces an increased expression of the LDL-cholesterol-receptor in hepatocytes leading to an uptake of these lipoproteins from the plasma. This mechanism leads to mean plasma LDL reduction of

35 to 55 percent at the maximal dose of different statins (Tobert, 2003). Statins are also effective in reducing the risk of cardiovascular events. A meta-analysis on five different statins considering 18 clinical trials found a 26% reduction in the risk of cardiovascular events compared with placebo. Said in another way, the treatment of 1000 patients with a statin can prevent 37 cardiovascular events (Silva et al., 2006). However, this is not true for all patients, as for example end-stage renal disease patients did not show a net increase in survival, although their LDL cholesterol levels were lowered according to guidelines for this patient group (4-D study on atorvastatin; Wanner et al., 2005)

1.3 Atorvastatin, its metabolism and possible links to myopathy

Statins are generally well tolerated drugs, though one to five percent of patients were found to develop muscle pain symptoms, which are reversible on discontinuation of the statin (Thompson et al., 2003). Severe adverse events are rare, but have been reported. A symptom called myopathy resulting mainly in muscle-pain and -weakness was observed. In some rare cases, myopathy patients developed rhabdomyolysis. This rapid muscle cell lysis releases breakdown products, some of which are harmful to the kidney and which may lead to kidney failure. A systematic review observed 3.4 cases of rhabdomyolysis in the same cohort studies per 100,000 person-years, of which 10 percent were fatal (Law and Rudnicka, 2006). A comprehensive explanation for the development of the described adverse events is not found yet. Plausible mechanisms describe it as a result of the inhibition of an early step of the cholesterol biosynthesis reducing not only the amount of produced cholesterol but also the production of mevalonate pathway products like ubiquinone (Coenzyme Q10) or isoprenoids/terpenoids (Marcoff and Thompson, 2007). But an involvement of the decreased farnesyl and geranyl-geranyl pyrophosphate production in the cholesterol-independent pleiotropic effects of statins was also suggested (Liao and Laufs, 2005).

An interesting observation has been that the incidence number of adverse events was higher in lovastatin, simvastatin and atorvastatin receiving patients (Law and Rudnicka, 2006). Additionally, a meta-analysis of 18 clinical trials observed that some statins are more prone to adverse events and atorvastatin was associated with the greatest risk of adverse events (Silva et al., 2006). This was further emphasized by reports of increased numbers of adverse events when statins are co-administered with known inhibitors of statin metabolizing or transporting proteins. Examples are itraconazole or ritonavir by inhibition of CYP3A4, or cyclosporine by inhibition of CYP3A4, P-glycoprotein and OATP1B1 (Neuvonen et al., 2006). A striking

example is the fibrate gemfibrozil where combinations with cerivastatin greatly increased the rate of rhabdomyolysis (Neuvonen et al., 2006) due to the inhibitory effect of gemfibrozil and gemfibroziol-glucuronide metabolite on CYP2C8 and OATP1B1 dependent metabolism and transport, respectively (Shitara et al., 2004). Cerivastatin was withdrawn from the market after an unusually high number of rhabdomyolyses had occurred in the United States.

Considering atorvastatin, a statin, which is highly metabolized and was the top-selling branded drug in the world in the year 2008 ("Financial Reports | Pfizer: the world's largest research-based pharmaceutical company," 2008), some interesting observations on certain metabolites and adverse events or toxicity were made recently. Hermann et al., (2006) compared the pharmacokinetics of atorvastatin and its metabolites in healthy controls and myopathy patients and found 2.4-fold and 3.1-fold increased AUC values for atorvastatin-lactone and p-OH-atorvastatin, respectively, in the myopathy patients. Additionally, when comparing the myotoxic potency of atorvastatin, it was found that atorvastatin-lactone had a 14-fold higher potency to induce myotoxicity in primary skeletal muscle cells than atorvastatin-acid (Skottheim et al., 2008). An involvement of metabolites in the development of adverse events like myopathy or rhabdomyolysis seems, therefore, likely for atorvastatin, and possibly also for the other statins.

Atorvastatin, together with rosuvastatin, is the most effective statin in lowering LDL-cholesterol. It showed a bioavailability of 12% and a high protein binding like all lipophilic statins (98%). The elimination half-life of the parent compound was found to be 14 hours (Schachter, 2005). Figure 1 summarizes the metabolic pathway of atorvastatin in human liver.

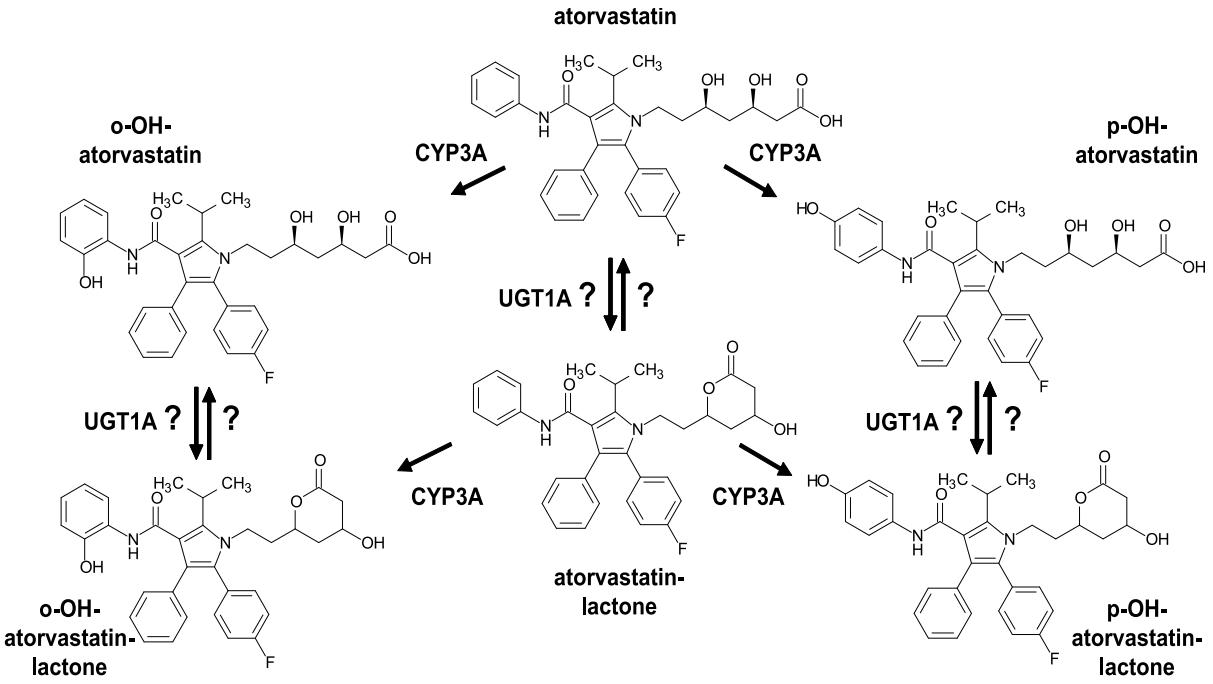


Figure 1: Oxidative and lactonization pathways of atorvastatin in human liver.

Atorvastatin is administered as an active drug in the statin-acid conformation, but like many statins it is also found in its inactive lactone conformation with nearly equal AUCs (Kantola et al., 1998). Both, the acid and the lactone conformation are substrates of CYP3A4 and CYP3A5 resulting in p- and o-OH-metabolites. A comparison of the metabolism of acid and lactone revealed that the lactone had a 3-fold higher V_{max} for p-OH-metabolite formation (identical V_{max} for o-OH-metabolites) and a 20-fold lower K_m, possibly due to higher lipophilicity, resulting in better access to the hydrophobic substrate channel of the CYP enzyme near the membrane surface. These results clearly indicated that the major elimination pathway of atorvastatin was via hydroxylation of atorvastatin-lactone and not via hydroxylation of the parent compound (Jacobsen et al., 2000). This brings into focus the aforementioned conversion between atorvastatin-acid and atorvastatin-lactone. Conversion to the lactone has been reported to occur spontaneously at low intestinal pH (Kearney et al., 1993) and probably mainly enzymatically at physiological pH (Goosen et al., 2007). Enzymatic formation was predicted to occur via a coenzyme A-dependent pathway (Li et al., 2006) and shown to occur via acyl-glucuronidation followed by spontaneous conversion to the lactone (Prueksaritanont et al., 2002). UDP-glucuronosyltransferase enzymes with known ability to this conversion are UGT1A1, 1A3 with minor contributions of 1A4, 1A8 and 2B7 (Goosen et al., 2007).

Hydrolysis of the lactone to the open acid conformation may also happen chemically or enzymatically via esterases or paraoxonases. Such a conversion by paraoxonase 1 has been shown for simvastatin or lovastatin (Billecke et al., 2000) and by paraoxonase 3 for lovastatin (Draganov et al., 2005) but a hydrolysis of atorvastatin-lactone by these enzymes apparently has not yet been examined.

1.4 Genes and genetic variation linked to atorvastatin metabolism

Why do some patients develop adverse events in statin treatment while most others do not? Possible explanations are inter-individual differences in the enzymes or transporters involved in the metabolism of the statin. In atorvastatin, as reported above, oxidative metabolism is mainly catalyzed by CYP3A4 and CYP3A5. CYP3A4 is one of the most important enzymes in oxidative hepatic metabolism (Zanger et al., 2008) and shows considerable inter-individual variability in its activity. Additionally, a sex-dependant difference with 50% higher activity resulting from increased enzyme expression in females was observed (Wolbold et al., 2003). But CYP3A4 seems not to be subject to common polymorphisms resulting in the absence of activity (Daly, 2010). Additionally only one common upstream polymorphism (*CYP3A4*1B*; -392A>G; rs2740574; VAF=2.5% in Caucasians) has been reported where one study could show a significantly lowered metabolic activity (Rodríguez-Antona et al., 2005). CYP3A5 is only expressed in approximately 10% of Europeans (Daly, 2006). An allele common in all studied populations (*CYP3A5*3*; rs776746; VAF=94.2% in Caucasians) creates a cryptic splice site resulting in the incorporation of intron sequence to the mature mRNA, which is then translated to a truncated protein. This explains the absence of CYP3A5 expression in many individuals (Kuehl et al., 2001) and contributes to the high inter-individual variability in CYP3A activity and may influence together with the *CYP3A4* variability the individual atorvastatin hydroxylation.

Furthermore of interest is a variation in the *SLCO1B1* gene coding for the statin uptake transporter OATP1B1. A genome-wide association study identified the *SLCO1B1* genotype as a predictor of susceptibility for statin-induced myopathy (Link et al., 2008). Independent studies have shown that this non-synonymous variation *SLCO1B1*5* (rs4149056; VAF=16.2% in Caucasians) affects the pharmacokinetics of several statins including atorvastatin (Pasanen et al., 2007, Voora et al., 2009, Niemi et al., 2011). Efflux transporters are also known to be involved in the transport of atorvastatin and its metabolites. A common variation in *ABCG2* resulting in an amino acid change (Gln141Lys; rs2231142; VAF=11.7%

in Caucasians) leading to reduced transport activity was found to be associated with significantly increased AUC values in carriers (Keskitalo et al., 2009). On the other hand, taking into account that neither OATP1B1 nor ABCG2 are expressed in skeletal muscles (Knauer et al., 2010) where the toxic effects of statins or their metabolites are observed other factors have to be considered.

1.4.1 Variability of UDP-glucuronosyltransferases possibly influencing atorvastatin-lactonization

Atorvastatin-lactonization is catalyzed via a glucuronide intermediate and an involvement of UGT1A1 and UGT1A3 in this reaction has been shown already by Prueksaritanont et al., (2002). These enzymes belong to the family of UDP-glucuronosyltransferases (UGTs), which are central phase II drug metabolizing enzymes (Tukey and Strassburg, 2000). Until today, 19 UGT proteins were identified in humans, which have been divided into three subfamilies, UGT1A, UGT2A, and UGT2B (Mackenzie et al., 2005). In addition to the glucuronidation of atorvastatin-acid, they catalyze the glucuronidation of a broad range of drugs and endobiotics. UGT1A1 for example is the only enzyme responsible for the elimination of the heme metabolite bilirubin (Tukey and Strassburg, 2000), and UGT1A3 was found to catalyze the glucuronidation of estrogens. UGT1A3 is also involved in the metabolism of bile acids like chenodeoxycholic and lithocholic acid to form C24-ester glucuronides. Though under physiological conditions C24 is mostly conjugated with glycine or taurine, under cholestatic conditions UGT1A3 mediated glucuronidation was found to be increased (Trottier et al., 2010).

The *UGT1A*-locus is located on chromosome 2q37 and consists of multiple first exons and shared exons 2-5 (figure 2). Recently, it has been shown that alternative splicing of the terminal exon 5 is leading to the expression of inactive UGT1A isoforms, which may possibly modulate the activity of the active UGT1A isoforms (Bellemare et al., 2011).

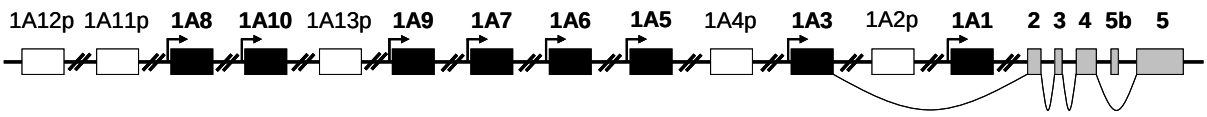


Figure 2: *UGT1A*-locus on chromosome 2q37. UGT1A isoforms are generated by alternative splicing of first exons to common exons 2 to 5, as shown above for UGT1A3. Five pseudogenes are not translated (*1A2p, 1A4p, 1A11p, 1A12p, 1A13p*).

UGTs are mainly expressed in liver, though some are found only in gastrointestinal tissue (UGT1A7, 1A8 and 1A10). Quantitative expression studies have only been performed at mRNA level due to a lack of selective antibodies. However, five members of the UGT1A family (UGT1A1, 1A3, 1A4, 1A6 and 1A9) and five members of the UGT2B family (2B4, 2B7, 2B10, 2B15 and 2B17) were found to be expressed more than 1% of total UGTs and also found to exhibit substantial inter-individual variability in their expression (Court, 2010, Ohno and Nakajin, 2009). For example, the expression of UGT1A1 and UGT1A3 were found to vary highly in a range of 0.9-138.5 (UGT1A1) and 0.1-66.6 (UGT1A3) $x10^4$ copies per µg mRNA in a selection of 25 livers (Izukawa et al., 2009).

One known important factor influencing the variability of *UGT1A1* is a common promoter variation (*UGT1A1*28*; rs34815109; VAF=34.1% in Caucasians) inserting an additional TA in the TATA-Box of the *UGT1A1* promoter reducing significantly the expression of UGT1A1. This was observed in people with Gilbert's Syndrom having moderately increased serum bilirubin levels. UGT1A3 variability was so far only examined in recombinant systems and several haplotypes were identified. One group tried to group them according to their activity, based on activity measurements of estrone glucuronidation with HEK293 cell lines stably expressing each haplotype (high: **1, *2, *3*; intermediate: **5, *7, *9, *10, *11*; low: **4, *6,*8*) (Caillier et al., 2007). Results from a group investigating the glucuronidation of flavonoids in a different expression system (baculovirus) did not reproduce these findings and on the contrary found **2, *3* and **5* to have low activity and **4* to have high activity.

1.4.2 Variability of esterases and paraoxonases and their possible involvement in atorvastatin-lactone hydrolysis

Atorvastatin-lactone hydrolysis may be catalyzed by several enzymes that are known to possess esterase activity (Testa and Mayer, 2003). One example is the group of carboxylesterases, of which CES1, 2 and 4 are known to be expressed in human liver (Yamada et al., 2010). An involvement of paraoxonases is also plausible (see 1.3). The three members of this family, PON1, PON2 and PON3 share an amino acid identity of ~65%. The PON1 structure consists of a six-bladed beta-propeller containing two Ca^{2+} ions necessary for enzyme stability and activity (Draganov, 2010). The activity of PON3 was also found to be Ca^{2+} dependent (Lu et al., 2006). Primarily, paraoxonases are lactone hydrolyzing enzymes (Draganov, 2007, Khersonsky and Tawfik, 2005). PON1 is known to hydrolyze toxic oxon metabolites of organophosphorous insecticides, also the nerve agents sarin and soman (Davies

et al., 1996), aromatic esters, as well as, phenylacetate and paraoxon, which are used as probe substrates. PON3 shares some lactone substrates with PON1 and catalyzes the hydrolysis of spironolactone and lovastatin (Draganov et al., 2005). PON2 was found to catalyze the hydrolysis of Dihydrocoumarin, which can also be hydrolyzed by PON1 and PON3 (Rosenblat et al., 2003).

The *PON*-locus is located on chromosome 7q21, where *PON2*, *PON3* and *PON1* follow one another, all of them transcribed in the same direction with intergenic regions of 8979bp between *PON2* and *PON3* and 35501bp between *PON3* and *PON1* (counted from exon to exon; figure 3).

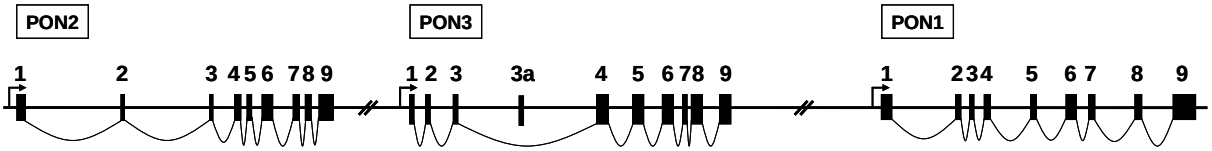


Figure 3: *PON*-locus on chromosome 7q21. Paraoxonases 1, 2 and 3 consist of 9 exons.

PON1 mRNA is only expressed in human liver, kidney and colon (Mackness et al., 2010). It is a secreted protein and found in the blood bound to HDL particles (Draganov, 2010). PON2 possesses a very wide tissue distribution (Ng et al., 2001, Mackness et al., 2010). PON3 was found to be expressed in liver, kidney and the gastrointestinal tract and thought to be secreted and HDL-bound like PON1. Recently, it has been postulated that due to a different N-terminal region, PON3 is not secreted in humans. Additionally, microsomal localization of PON3 was found in rabbit and rat livers (Draganov, 2007).

Inter-individual variability of PON1 was found to be 13-fold in human blood serum (Costa et al., 2003). Several polymorphisms were published affecting PON1 expression or activity. Within the promoter, a list of polymorphisms could be identified and one of them, *-108 C>T* (rs705379; VAF=38.9% in Caucasians), was described to disrupt a binding site for the transcription factor Sp1 and was associated with decreased expression (Deakin et al., 2003). Coding polymorphisms affecting the catalytic activity were described. Interesting observations were made on *Q192R* (rs662; VAF=35.8% in Caucasians). The PON1 isoform carrying *192Q* hydrolyzed paraoxon slowly and diazoxon, sarin and soman rapidly, whereas *192R* hydrolyzed paraoxon rapidly and diazoxon more slowly. No difference between the isoforms was found in the hydrolysis of phenylacetate. Due to a lack of selective protein

quantification methods, these differences were used to identify a "PON1 status" by measuring phenylacetate hydrolysis and one of the variant activities (Costa and Furlong, 2002).

Not much is known about the variability of PON3 and no association studies with PON3 expression data have been performed. Although several promoter polymorphisms were identified and used in an association study with PON1 substrates, their functional relevance remained unclear (Marsillach et al., 2009).

1.5 Objectives

Increased levels of atorvastatin-lactone found in myopathy patients are highly interesting in the terms of atorvastatin-related adverse events (Hermann et al., 2006). Atorvastatin-lactone is much more lipophilic than atorvastatin-acids, so that it should enter the muscle cells much easier. In addition to this, lactones had stronger myotoxic effects on primary skeletal muscle cells (Skottheim et al., 2008). Based on these observations, the starting hypothesis of this thesis is that inter-individual variations of enzymes involved in atorvastatin-lactonization or atorvastatin-lactone hydrolysis might be important risk factors for the development of adverse events in treatment with atorvastatin.

Therefore, crucial objectives of this work are to identify enzymes responsible for atorvastatin-lactonization and -lactone hydrolysis, in addition to the identification of cytochrome P450 enzymes involved in atorvastatin-hydroxylation. *In vitro* experiments from a population of liver samples will provide important information on inter-individual differences in enzyme expression and atorvastatin-lactone formation. Following this, genetic variability within the identified genes will be determined and analyzed for associations with enzyme expression and atorvastatin-lactone formation. In a similar approach, inter-individual differences in the expression of enzymes responsible for atorvastatin-lactone hydrolysis will be determined in the same population. Genetic variability in the corresponding genetic loci will be studied and used in associations with enzyme expression and atorvastatin-lactone hydrolysis.

In this way, genetic variability will be determined, possibly explaining increased levels of atorvastatin-lactone, a metabolite associated with toxicity. Finally, identified influential polymorphisms of atorvastatin-lactone levels will be tested in patient cohorts for their *in vivo* influence on atorvastatin pharmacokinetics, as well as, lipid levels and patient survival.

2. Results

2.1 Atorvastatin hydroxylation

Hydroxylation has an important role in the metabolism of atorvastatin (see 1.3). Atorvastatin-acid and -lactone are substrates for hydroxylation at the para- and ortho-position. The aim of the following experiments was to ascertain previous reports that atorvastatin is mainly hydroxylated by CYP3A4 and CYP3A5.

2.1.1 Identification of CYPs responsible for atorvastatin-hydroxylation

Incubations with 11 recombinantly expressed cytochrome P450 (CYP) enzymes at 10 and 100 µM atorvastatin showed that it is a highly specific substrate for CYP3A4 and CYP3A5. CYP2C8 exhibited minor p-OH-metabolite (but no o-OH metabolite) formation, which contributes however less than 2% of the total activity at a concentration of 100 µM atorvastatin. Comparing the formation of both hydroxy-metabolites, CYP3A4 had a 1.7-fold and CYP3A5 a 24.6-fold higher p-OH than o-OH-metabolite formation. The p-OH-atorvastatin formation was nearly identical in both CYP3A enzymes, whereas o-OH-atorvastatin was formed 13.5-fold more by CYP3A4 than by CYP3A5 (figure 4).

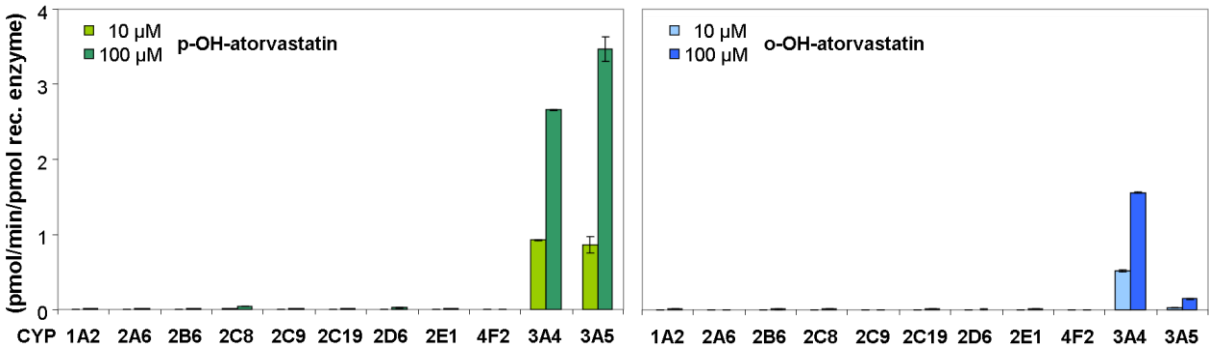


Figure 4: Hydroxy-metabolite formation of atorvastatin in 11 recombinantly expressed Cytochrome P450 Supersomes (25 µg) at substrate concentrations of 10 and 100 µM. Columns represent the mean of two measurements; standard deviation is marked by error bars.

2.1.2 Differential effect of cytochrome b5 co-expression on hydroxylation in CYP3A enzymes

Cytochrome P450 enzymes are monooxygenases receiving two electrons originating from NADPH via the CYP oxidoreductase (CYPOR). The second electron can also be delivered via cytochrome b5. Recombinantly expressed CYP enzymes used in these experiments are receiving their electrons from co-expressed CYPOR. Additional experiments were performed using co-expressed CYPOR and cytochrome b5. In this recombinant system, CYPOR and

cytochrome b5 were expressed in excess with ratios of 1:7:7 for CYP3A4:CYPOR:cytochrome b5 and 1:13:13 for CYP3A5:CYPOR:cytochrome b5. The additional electron donator cytochrome b5 increased the activity of CYP3A4 around 6-fold for o- and p-OH-atorvastatin and of CYP3A5 around 6- to 8-fold for ortho-OH-atorvastatin. Interestingly, the p-OH-metabolite formed by CYP3A5 was only increased 1.7-fold by co-expressed cytochrome b5 (figure 5).

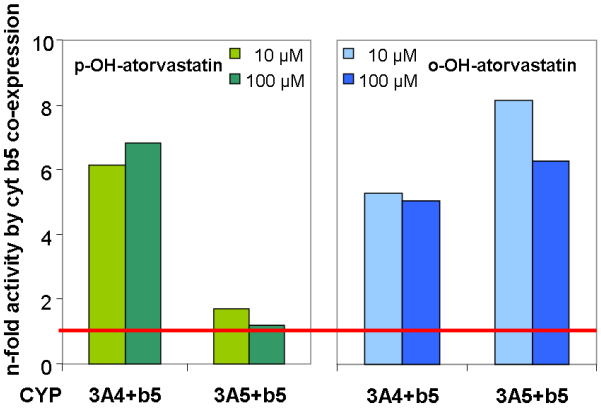


Figure 5: n-fold hydroxylation activity by co-expressed cytochrome b5 in recombinantly expressed CYP3A4 and CYP3A5 enzymes at atorvastatin concentrations of 10 and 100µM.

2.2 Atorvastatin-Lactone formation

Statins are administered either as active open acid (atorvastatin, pravastatin or rosuvastatin) or as inactive pro-drug in the form of a lactone (simvastatin or lovastatin) and, as mentioned earlier for atorvastatin, both forms of the drug can be found in the body due to interconversion between acid and lactone. Lactone formation may occur non-enzymatically at low intestinal pH (Kearney et al., 1993). In addition to this way, enzymatical conversion through an unstable acyl glucuronide (Prueksaritanont et al., 2002) or a coenzyme A intermediate (Li et al., 2006) have been described. Statin lactones, on the other hand, can be hydrolyzed chemically or enzymatically by esterases and paraoxonases (Vickers et al., 1990, Billecke et al., 2000).

2.2.1 General role of UGTs in statin-lactone formation

To estimate the importance of the formation of lactone or acid of the different statins and later on to identify the responsible pathway, it was of interest to determine the relative amount of lactone and acid in plasma. A screening of literature was performed to estimate the plasma-levels of acid and lactone for five statins (figure 6).

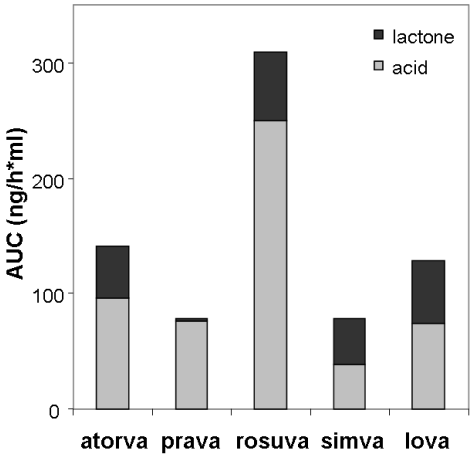


Figure 6: Summary of acid and lactone AUC values from several studies in healthy volunteers having received 10-80 mg statins in single or multiple dosing.

Pravastatin, which is given to the patient as an open acid was only minimally converted to lactone. Simvastatin exhibited equal amounts of both forms of the drug and lovastatin was also found to have high statin interconversion. Atorvastatin and rosuvastatin on the other hand showed lactone AUCs of up to 47% or 24% of the corresponding statin-acid AUC (Lilja et al., 1999; Kantola et al., 1998; Rogers et al., 1999; Ziviani et al., 2001; Bucher et al., 2002; Fukazawa et al., 2004; Schneck et al., 2005; Lee et al., 2005; Becquemont et al., 2007; Choi et al., 2008). Thus, lactone formation was obviously a major pathway for atorvastatin and rosuvastatin, therefore, a possible enzymatic involvement in these reactions was investigated. Conversion of statin-acid to lactone was described to be mediated by UDP-glucuronosyl-transferases (UGTs) (Goosen et al., 2007) via an unstable statin-glucuronide intermediate, which is spontaneously converted to the statin-lactone. The aim of the following experiments was to determine the role of this possible biotransformation in the lactonization of various statins. Using pooled human liver microsomes, five statin-acids were incubated with and without the co-substrate UDP-glucuronic-acid needed for enzymatic activity of UGTs (figure 7).

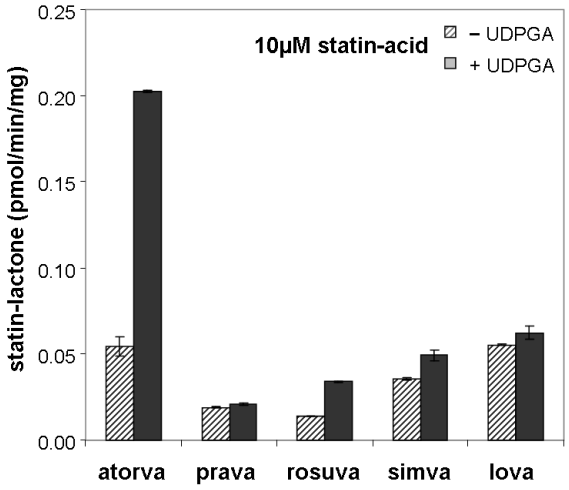


Figure 7: Statin-lactone formation from 10µM of the corresponding statin-acid with and without UDP-glucuronic acid (UDPGA) in 25µg pooled human liver microsomes. Shown is the mean of two measurements and standard deviations are assigned as error bars.

Simvastatin and lovastatin exhibited only minimal conversion from the open acid to the lactone form both with and without the co-substrate UDPGA. Corresponding to the data presented in figure 6, pravastatin was not converted to its lactone in an UDPGA dependent manner, but atorvastatin and rosuvastatin showed 3.7- and 2.4-fold higher lactone formations respectively, in the presence of UDPGA. Additionally, atorvastatin was found to have the highest lactone formation rate of all five statins. When compared with the hydroxylation of atorvastatin acid by recombinant CYP3A4 as shown in figure 4, lactonization in this experiment was ~1000-fold lower per mg protein. But this comparison must be judged with care, as no molar enzyme levels could be compared. Nevertheless, the observations in figure 7 demonstrate that UGT-dependent lactone formation is of potential importance in the biotransformation of atorvastatin.

2.2.2 Identification of UGTs responsible for atorvastatin-lactone formation

UDP-glucuronosyltransferases (UGTs) are phase II drug metabolising enzymes (Tukey and Strassburg, 2000). They catalyze the glucuronidation of a broad range of substrates (see 1.4.1) and previous reports had shown an involvement of certain UGTs in this reaction (Goosen et al., 2007). Hence, it was necessary to identify candidate UGTs important in atorvastatin-lactonization. A broad range of recombinantly expressed isozymes of the UGT1A and UGT2B group are commercially available for screening experiments and were selected to identify their importance in this reaction. Incubation conditions were principally derived from experiments previously described in literature (Goosen et al., 2007) and incubation time, as

well as, protein and substrate concentration were further optimized to be within the linear range of the activity of the enzyme. Quantification was performed by using a highly sensitive LC-MS/MS, which allowed the quantification of 0.5-500pmol of atorvastatin-lactone. The incubation of 9 recombinantly expressed UDP-glucuronosyltransferase enzymes with 1 and 10µM atorvastatin revealed significant atorvastatin-lactone formation only in UGT1A1 and UGT1A3. At the concentrations 1 and 10µM, the activity of UGT1A3 was 3.7- or 9.3-fold higher than in the UGT1A1 isozyme, respectively (figure 8).

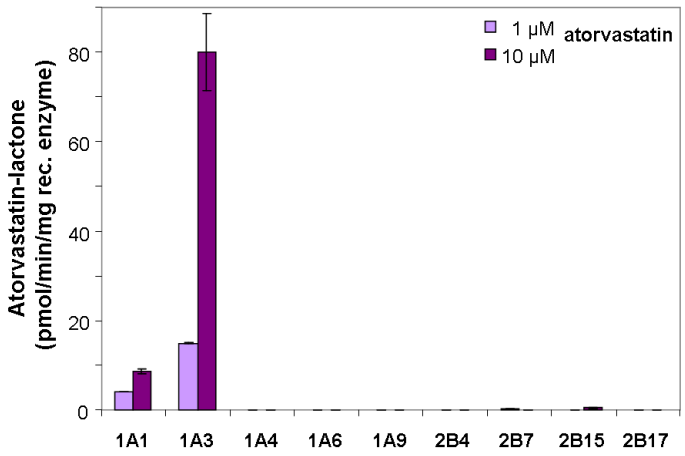


Figure 8: Atorvastatin-lactone formation from 1 and 10µM atorvastatin with 25µg of 9 recombinantly expressed isozymes of UDP-glucuronosyltransferases. Incubations were performed at 37°C for 30 minutes. Atorvastatin-lactone was quantified via LC-MS/MS. Bars represent the mean of two measurements and standard deviation is shown by error bars.

In vitro kinetic parameters of UGT1A1 and UGT1A3 are shown in figure 9. No maximal turnover rate could be reached for UGT1A1 within the range of solubility of atorvastatin in the chosen solute (acetonitrile/water). For UGT1A3 on the other hand a V_{max} of 212pmol/min/mg and a K_m of 12µM was determined, indicating that UGT1A3 is a highly efficient enzyme in atorvastatin glucuronidation (figure 9).

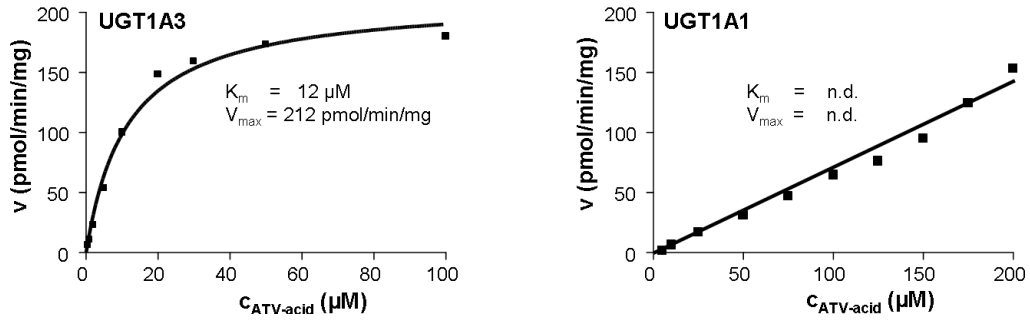


Figure 9: Determination of the kinetic parameters of the formation of atorvastatin-lactone in 25µg of recombinantly expressed UGT1A3 and UGT1A1.

UGT1A3 was therefore the most likely candidate for the formation of atorvastatin-lactone. Because the relative expression levels of UGT1A1 and 1A3 in liver were not known, an important role of UGT1A1 could not be excluded and investigations in human liver microsomes had to be performed to answer this question.

2.2.3 Population variability of microsomal atorvastatin-lactonization and UGT1A1 and UGT1A3 expression

Liver samples (N=150) from a clinically, as well as, genotypically and phenotypically well characterized Caucasian population were used to further investigate the relative contribution of UGT1A1 and UGT1A3 in atorvastatin-lactone formation. mRNA and protein expression of these enzymes were analyzed, as well as, the atorvastatin-lactone formation microsomal preparations of these livers.

The mRNA expression was detected in cDNA generated from extracted high quality total RNA. Quantitative real-time PCR (Taqman) was performed to quantitate UGT1A3 mRNA expression using highly specific UGT1A3 primers spanning exons 1 to 3 to avoid co-detection of genomic DNA and of UGT1A4 or UGT1A5, which have more than 97% mRNA identity with UGT1A3. UGT1A1 mRNA quantitative data were obtained from the Human-6 v3 Expression BeadChips (Illumina, Eindhoven, Netherlands). The UGT1A1 probe was localized in exon 1 differing from UGT1A3, 1A4 or 1A5 in 11 of 50 bases of the probe.

Protein expression of UGT1A1 (performed by Christan Klenner) and UGT1A3 was relatively quantified by western blot analysis on microsomal preparations available from all samples. Both enzymes have highly variable expression levels not related to each other (figure 10). Whereas UGT1A1 was identified as a 54kDa enzyme, as shown previously by Kerdpin et al., (2009), UGT1A3 appeared as a double band at 54/57kDa, which also occurred in recombinant UGT1A3, where an additional band at 52kDa was detected. Endoglycosidase treatment was performed for the recombinant enzyme and human liver microsomes to test whether the bands reflected various states of glycosylation. Indeed deglycosylation lead to a shift of the detected bands towards a single 50kDa band in human liver microsomes and a 50/52kDa band for the recombinant enzyme. This finding suggests UGT1A3 to be an entirely glycosylated protein in the liver, which is in accordance to previously detected glycosylation sites of UGT1A1 (N102) and 1A3 (N142) (Chen et al., 2009).

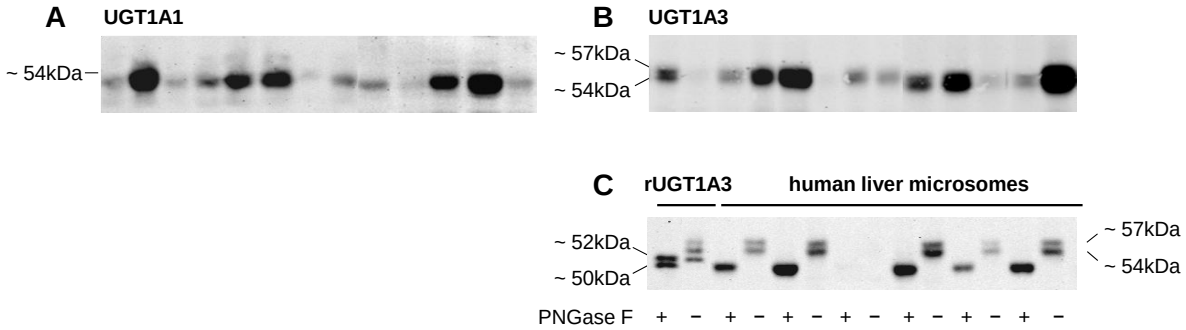


Figure 10: Immunoblots of human liver microsomes stained with specific antibodies against UGT1A1 (A) and UGT1A3 (B). Deglycosylation was performed by analysis of recombinant UGT1A3 (Supersomes) or human liver microsomes with (+) or without (–) pre-treatment with endoglycosidase PNGase F (C). Relative molecular weight was determined using marker proteins.

Microsomal activity of UGTs was determined by incubation experiments with human liver microsomes (identical to conditions applied in 2.2.2) of this population (N=150). Figure 11 shows the high variability of the formation of atorvastatin-lactone in the Caucasian population. Lactone formation ranged from 2.7 to 52.6pmol/min/mg.

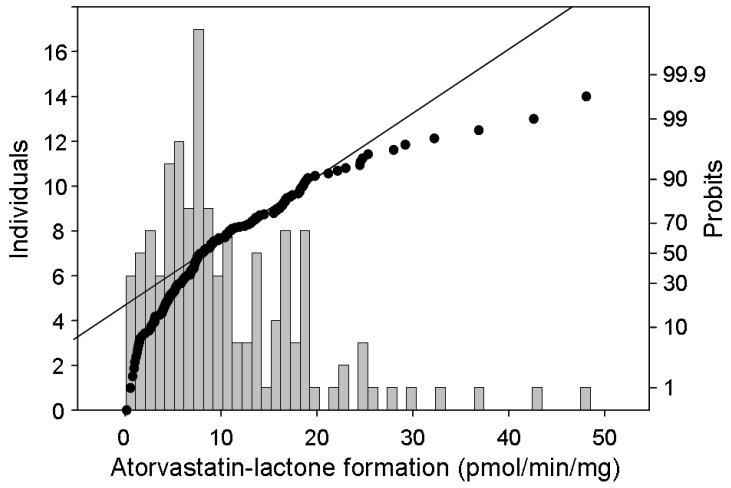


Figure 11: Frequency histogram (left axids) and cumulated frequency plot (right axis) showing population distribution of atorvastatin-lactone formation from 10µM atorvastatin-acid (N=150). Incubations (25 µg of microsomal protein) were performed in the presence of UDPGA and alamethicin at 37°C for 30 minutes. atorvastatin-lactone was quantitated by LC-MS/MS analysis.

A comparison of the variability (table 1) in expression and lactonization-activity revealed that mRNA of UGT1A3 and protein levels of UGT1A1 and UGT1A3 were comparable ranging between 87% and 102% when coefficients of variation were compared. UGT1A1 mRNA showed only minimal variability, which can be explained by the fact that these $\log_2$ data were derived from Human-6 v3 Expression BeadChips (Illumina, Eindhoven, Netherlands). When

UGT1A3 mRNA data is transformed to log_2, the coefficient of variation of 19% is in a similar range to the UGT1A1 coefficient (9%). Atorvastatin-lactone formation was only 62% variable.

Table 1: Population variability of hepatic PON1 and PON3 expression phenotypes.

	UGT1A1		**UGT1A3**		**Atorvastatin-lactonization** (pmol/ min/mg)
	mRNA/ quantil norm. relative units	protein (µg/µl)	mRNA/ RPLP0 relative units	protein (µg/mg)	
Minimum	6.42	0.03	0.0010	1.58	2.73
Median	8.46	0.86	0.0095	82.63	10.83
Maximum	10.66	4.63	0.0669	837.7	52.63
Ratio max./min.	1.66	154.33	69.19	530.19	19.28
Normal distribution	No	No	No	No	No
Coefficient of variation (cv, %)	9	87	90	102	62

These generated datasets on the expression of UGT1A1 and UGT1A3 and on the activity in the transformation of atorvastatin-acid were then used for correlation analyses. Table 2 summarizes the significance of the correlation experiments (***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$) and the corresponding Spearman coefficient (r_s). As expected, UGT1A1 and UGT1A3 mRNA correlated well with their protein data. However, only UGT1A3 mRNA and protein correlated significantly with the formation of atorvastatin-lactone, whereas UGT1A1 protein correlated with lactone formation only with a Spearman coefficient of 0.2, suggesting that this reaction may be catalyzed mainly by UGT1A3. Surprisingly, UGT1A1 mRNA also correlated negatively with UGT1A3 mRNA and protein, but with Spearman coefficients of -0.25 or -0.41 respectively, a fact, which may point to some genetic relation between the two enzymes.

Table 2: Correlation analysis of UGT1A1 and UGT1A3 expression and atorvastatin-lactone formation.

UGT1A1 protein	UGT1A3 mRNA	UGT1A3 protein	ATV-lactone formation	
*** $r_s = 0.51$	** $r_s = -0.25$	*** $r_s = -0.41$	n.s.	UGT1A1 mRNA
	n.s.	n.s.	* $r_s=0.20$	UGT1A1 protein
		*** $r_s = 0.62$	*** $r_s = 0.53$	UGT1A3 mRNA
			*** $r_s = 0.61$	UGT1A3 protein

2.2.4 Genetic variability of *UGT1A3*-locus and *UGT1A1*28* in a Caucasian population

High variability within biotransformation pathways is influenced by environmental and also genetic factors. One reason for drug induced toxicities in certain patients can be an extremely high or extremely low production of certain metabolites in patients. Therefore, the observed high variability in the formation of atorvastatin-lactone might be an important observation concerning the development of atorvastatin induced adverse effects.

As atorvastatin-lactone formation is catalyzed by UGT1A3, variability in this activity may be determined by genetic variability within the *UGT1A*-locus. This locus is characterized by the fact that the UGT1A isozymes are generated by alternative splicing, which means that they differ only in exon 1 and share the common exons 2-5 (see 1.4.1). The *UGT1A1*28* polymorphism (rs8175347) was chosen for genotyping as it is known to be a major influence factor for UGT1A1 expression (Bosma et al., 1995). Polymorphisms for genotyping of *UGT1A3* exon 1 were chosen as described in the literature (Caillier et al., 2007). The development of a MALDI-TOF MS- based genotyping assay for UGT1A3 had to take into account the high homology of *UGT1A3* exon 1 with other members of the UGT1A-family. Whereas the exon 1 homology to *UGT1A1* was 63%, *UGT1A4* and *UGT1A5* share 93% and 94% of their exon 1 sequence with *UGT1A3*. Therefore, the position of the primers for the generation of pre-amplification fragments spanning all areas for SNP detection, were chosen carefully to be *UGT1A3* specific. Additionally, a touchdown PCR was performed to increase the specificity of the amplification. An advantage of the MALDI-TOF MS- based sequencing technology is the possibility to determine polymorphisms in multiplex assays. Therefore, it was possible to determine all 19 *UGT1A3* SNPs of the genomic DNA of this Caucasian

population (N=150) in a 12- , a 4- and a 3-plex assay. Table 3 summarizes the allelic frequencies of the variant allele (VAF) of this population. Variant allelic frequencies of all SNPs were comparable to frequencies of the dbSNP database, where the information was available. The allelic distributions of all polymorphisms detected in this population were tested for, and did not deviate from, Hardy-Weinberg equilibrium.

Table 3: Characteristics and variant allele frequencies (VAF) of *UGT1A3* SNPs determined in the IKP liver bank, 100 samples from a healthy volunteer study conducted in Finland (ATOGEN) and the dbSNP database (build 132).

SNP	*SNP ID*	*Genomic position*	*Base change*	*Residue change or promoter position*	*Region*	*VAF IKP liver bank*	*VAF atogen study*	*VAF dbSNP Build 132*
1	rs2008584	144977	A>G	-758	promoter	0.457	0.480	0.5
2	rs1983023	144984	T>C	-751	promoter	0.397	0.440	0.336
3	rs2008595	145154	C>T	-581	promoter	0.457	0.480	0.458
4	rs45507691	145182	G>A	-553	promoter	0.057	0.020	0.058
5	rs3806597	145587	A>G	-204	promoter	0.457	0.474	0.499
6	rs3806596	145669	T>C	-66	promoter	0.457	0.480	0.451
7	rs28898617	145751	A>G	Q6R	exon 1	0	-	0
8	rs3821242	145765	T>C	W11R	exon 1	0.457	0.480	0.458
9	rs6706232	145815	G>A	E27	exon 1	0.457	0.480	0.457
10	rs6431625	145874	T>C	V47A	exon 1	0.397	0.440	0.367
11	rs17868336	145968	A>G	T78	exon 1	0.033	0.020	0.05
12	not available	146062	T>A	F110I	exon 1	0	0	-
13	rs28898619	146076	G>A	M114I	exon 1	0	0	0
14	rs61764030	146207	C>T	A158V	exon 1	0.007	0	-
15	rs7574296	146211	A>G	A159	exon 1	0.457	0.480	0.458
16	rs45586035	146253	G>-	frameshift	exon 1	0	0	0.007
17	not available	146271	T>C	D179	exon 1	0	0	-
18	not available	146356	A>C	M208L	exon 1	0.003	0	-
19	rs45449995	146542	A>G	M270V	exon 1	0.050	0.020	0.014
20	rs8175347; *UGT1A1*28*	176861	$(TA)_{6/7}$	-40/-41	1A1 promoter	0.324	-	0.335

Additionally, pairwise linkage disequilibrium values were calculated for these SNPs (figure 12). A single haplotype block of 31kb was identified comprising all detected SNPs in this population. Highest r^2 values were found for 5 promoter, two non-synonymous (W11R, V47A) and two synonymous (*E27*; rs6706232, *A159*; rs7574296) SNPs, which were also found to be in high linkage with *UGT1A1*28*.

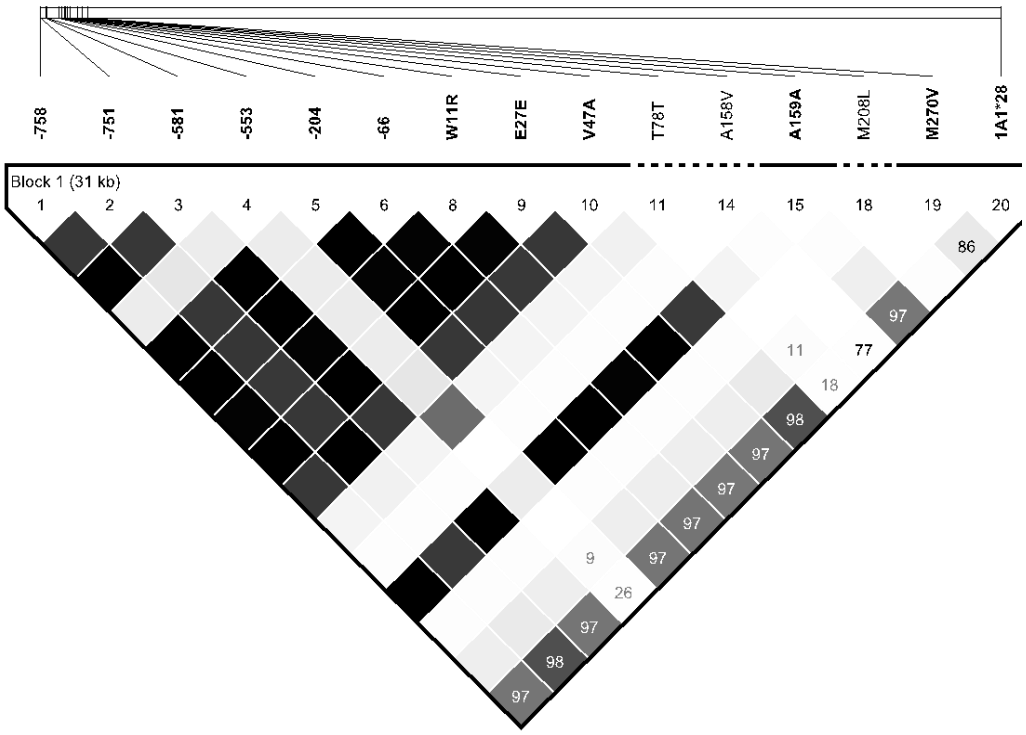


Figure 12: Haplotype map of the *UGT1A*-locus generated by Haploview 4.1 from data related to 15 SNPs detected in the livers. Pairwise linkage disequilibrium values (r^2 values) are indicated in different shades of gray. As indicated by the bold line, the entire region comprising 31kb represents a single haplotype block.

2.2.5 Identification of UGT1A-haplotypes

It was possible to associate all except one of the detected polymorphisms to previously described haplotypes ("Nomenclature: UDP Glucuronosyltransferase Alleles," 2011). A new haplotype **12* was deduced for one sample carrying the M208L variation. **2* alleles were most prominent in this population with a predicted frequency of 0.34 for the three subtypes. They are characterized by carrying 5 promoter polymorphisms, as well as, the W11R (rs3821242) and V47A (rs6431625) and the synonymous variations E27 (rs6706232) and A159 (rs7574296). Sub-haplotypes **2c* and **2d* differ by carrying an additional promoter SNP (*-553*) and **2c* carrying also the T78 (rs17868336) variation.

Additionally the **3* and **6* alleles occurred with frequencies of 0.06 and 0.05, respectively. **3* differs from **2a* by missing the *-751T>C* promoter and V47A (rs6431625) variations, whereas *6 is identical to **2a* with the additional M270V (rs45449995) amino acid exchange. The rare alleles **12* and **8* were only identified in one or two heterozygote carriers, respectively. **12* is also identical to **2a* with the additional M208L variation and **8a* is identical to a wild-type allele carrying the A158V (rs61764030) variation.

Table 4: Haplotypes deduced from genotyping a Caucasian population (N=150) for polymorphisms in UGT1A3 and their predicted frequencies.

SNP no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
Position/ Function	-758	-751	-581	-553	-204	-66	Q6R	W11R	E27E	V47A	T78T	F11I	M114I	A158V	A159A	frameshift	D179D	M208L	M270V	*Predicted Frequency*
UGT1A3*1a	**A**	**T**	**C**	**G**	**A**	**T**	**A**	**T**	**G**	**T**	**A**	**T**	**G**	**C**	**A**	**G**	**T**	**A**	**A**	**0.537**
UGT1A3*2a	G	C	T		G	C		C	A	C					G					**0.287**
UGT1A3*2c	G	C	T	A	G	C		C	A	C	G				G					**0.033**
UGT1A3*2d	G	C	T	A	G	C		C	A	C					G					**0.023**
UGT1A3*3a	G		T		G	C		C	A						G					**0.060**
UGT1A3*6a	G	C	T		G	C		C	A	C					G				G	**0.050**
UGT1A3*8a														T						**0.006**
UGT1A3*12	G	C	T		G	C		C	A	C					G			C		**0.003**

As mRNA expression of UGT1A3 was shown previously for several cell-lines (Nakamura et al., 2008), genotypes of two colon carcinoma and four liver derived cell lines were determined. Huh7 were found to be homozygotic *UGT1A3*1a*, Caco2 homozygotic **2a* and the other tested cell lines **1a/*2* carriers (table 5).

Table 5: *UGT1A3* genotypes of several important colon and liver cell lines.

cell line	genotype
CaCo2	**2a/*2a*
IHH	**1a/*2a*
LS174	**1a/*2a*
HepG2	**1a/*2a*
HepaRG	**1a/*2c*
Huh7	**1a/*1a*

2.2.5.1. Influence of UGT1A1*28 and UGT1A3-haplotypes on enzyme expression and activity

Associations of the identified genetic variations were then used for analyzing activity and expression differences in atorvastatin-lactonization and UGT1A1 or UGT1A3 expression (figure 13). The *UGT1A1*28* genotype is described as a polymorphism with clinical consequences as it is associated with decreased UGT1A1 expression resulting in elevated bilirubin levels in serum referred to as the Gilbert's Syndrome (Johnson et al., 2009).
An analysis of the influence of *UGT1A1*28* genotype revealed an interesting association on UGT1A1 expression on one hand and lactonization on the other hand. Whereas UGT1A1 mRNA expression was reduced 0.67-fold for heterozygous and 0.39-fold for homozygous **28* carriers and protein expression was reduced 0.66-fold and 0.33-fold, respectively, lactonization was increased in a **28* dependent manner (1.29-fold in heterozygous and 1.93-fold in homozygous carriers). In analogy to this, UGT1A3 protein expression was increased even more (3.0 in heterozygous and 5.0-fold in homozygous carriers). Taking into account the high linkage between *UGT1A1*28* and polymorphisms within exon 1 of UGT1A3, this fact also supports the idea that UGT1A1 is not the main enzyme catalyzing atorvastatin-lactonization.

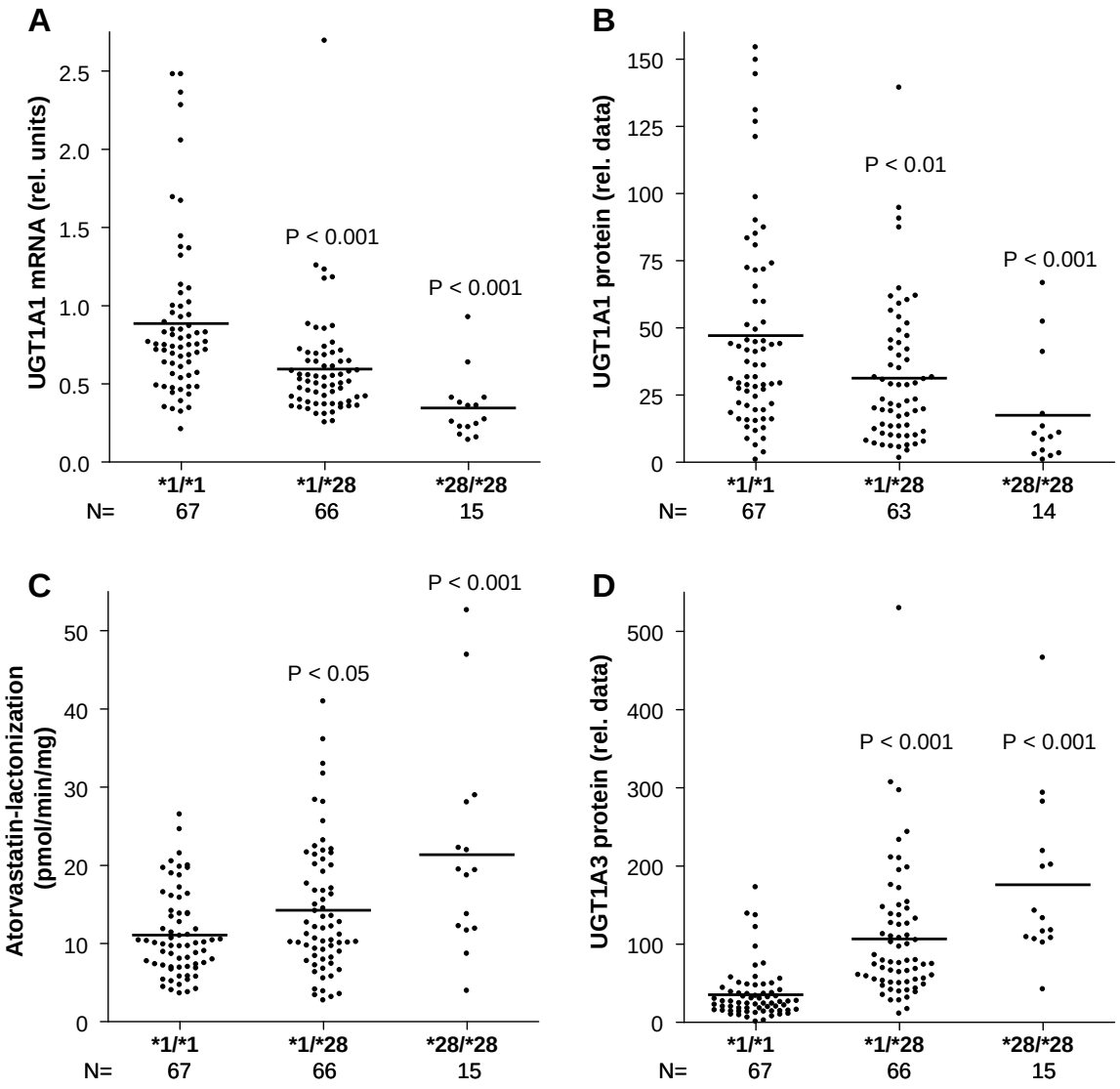


Figure 13: Influence of *UGT1A1*28* polymorphism on phenotypes. Median of UGT1A1 protein, mRNA, and microsomal atorvastatin δ-lactonization activity in liver microsomes were calculated for the indicated *UGT1A1*28* promoter polymorphism genotypes. Plots indicate the median (horizontal line) and statistical significance as compared with wild-type (**1/*1*) is given.

Seeing this surprising inverse relation between UGT1A1 and 1A3 when analyzed for *UGT1A1*28* it was of high interest to analyze the activity and expression data according to the predefined UGT1A3 haplotypes (figure 14). These analyses revealed that the most frequent variant haplotype, *UGT1A3*2,* was found to exhibit a clear genotype dependent trend towards significantly increased mRNA and protein expression reflecting the increased atorvastatin-lactonization. In **2* heterozygous and homozygous carriers, mRNA was increased 3.5- and 4.4-fold, protein level 4.5- and 7.3-fold, and atorvastatin-lactonization 1.4- and 2.1-fold. The less common haplotypes **3* and **6* only detected in heterozygous samples were not associated with significant changes in atorvastatin-lactonization, but increased

UGT1A3 mRNA 2.3- and 4.7-fold, as well as, protein level 1.4- (not significant) and 2.5-fold, respectively.

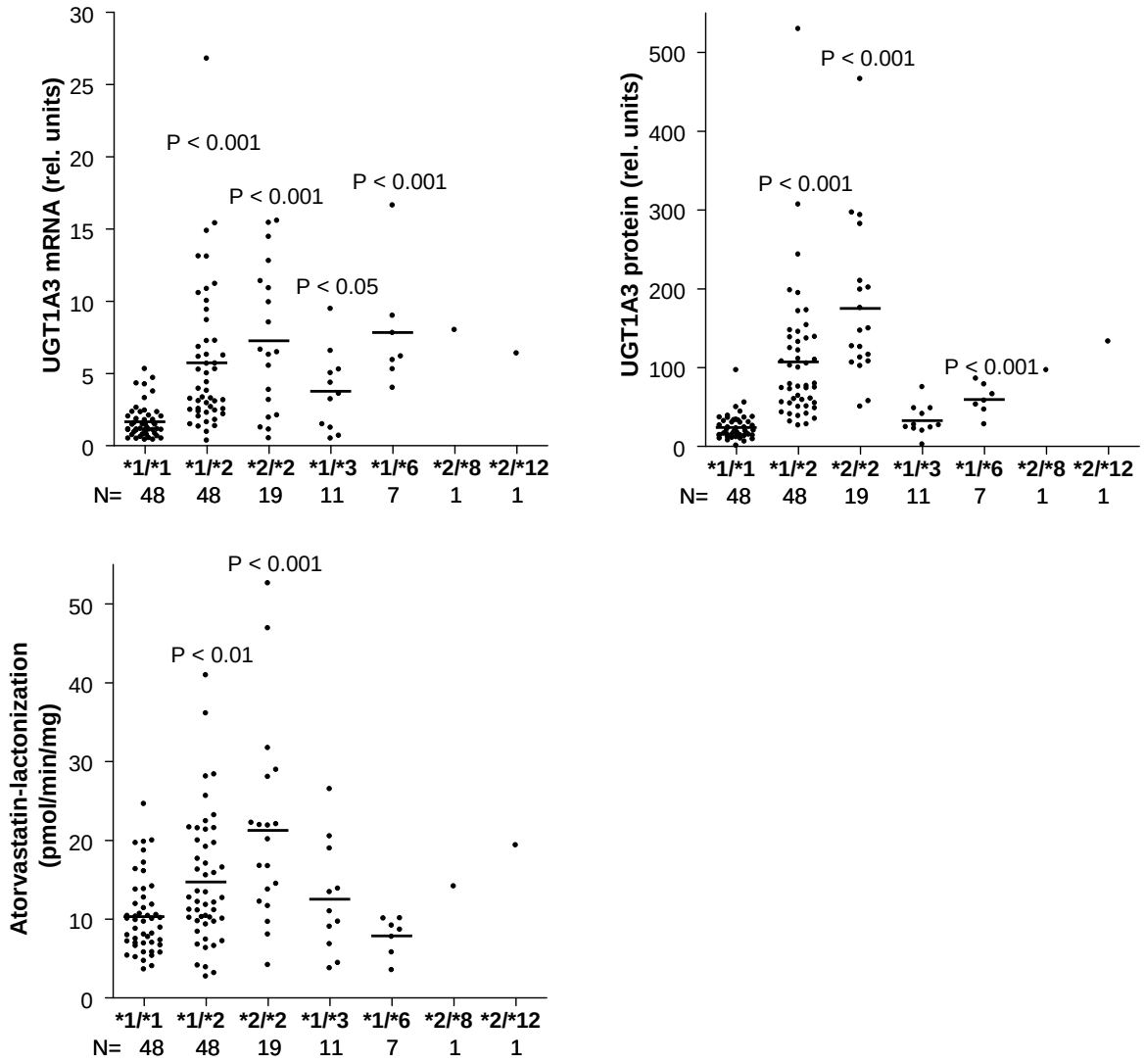


Figure 14: Influence of UGT1A3 haplotypes on phenotypes. Levels of UGT1A3 protein, mRNA, and microsomal atorvastatin δ-lactonization activity in liver microsomes were calculated for the indicated UGT1A3 haplotypes. Plots indicate the median (horizontal line) and statistical significance as compared with wild-type (**1/*1*) is indicated.

So, genotype or haplotype dependent stratification of activity and expression data of the Caucasian population revealed that atorvastatin-lactonization was inversely associated with UGT1A1 expression via *UGT1A1*28* and strongly associated with *UGT1A3*-haplotype **2*. *UGT1A3*2* was found to be linked with significantly higher UGT1A3 expression leading to significantly higher atorvastatin-lactonization compared to *UGT1A3*1*. This fact made the *UGT1A3*2* haplotype a likely candidate responsible for elevated atorvastatin-lactone levels in patient groups and also for a possible link to atorvastatin induced myopathy (see 1.3)

2.2.5.2 UGT1A3 and *UGT1A3*-haplotypes in the biotransformation of atorvastatin and three endogenous substrates (estrone, DCA, LCA)

UGT1A3 was not only described to be involved in the metabolism of atorvastatin, but was also described as a major inactivating pathway for estrone (Caillier et al., 2007) and bile acids (Barbier et al., 2006).

In the inactivation pathway of the estrogenic hormone estrone (E1) to estrone-3-glucuronide, UGT1A3 was known to be one of the most efficient enzymes. The alternative pathway, a sulphation of E1, leads to the most abundant estrogen precursors, which is a major source for active E2 in endometrial and mammary tissues (Caillier et al., 2007). In the biotransformation of bile acids, glucuronidation allows their transport via conjugate transporters at the basolateral membrane of hepatocytes, facilitating their secretion into blood followed by urinary excretion. Via this pathway, glucuronidated bile acids can escape the enterohepatic recirculation. Two of the six major bile acids were selected as probe substrates for this work. Deoxycholic acid (DCA) is produced from cholic acid whereas lithocholic acid is produced from chenodeoxycholic acid, and is also a direct precursor of the highly abundant bile acid hyodeoxycholic acid (Barbier et al., 2006). DCA and LCA are therefore reflecting two important ways of bile acid interconversion.

The aims of the following studies were to confirm the role of UGT1A3 within the described inactivating pathways, as well as, to detect possible influences of the previously identified UGT1A3 haplotypes on these glucuronidation pathways.

2.2.5.2.1 Biotransformation of atorvastatin, estrone, DCA and LCA by UGT-isoenzymes

Incubations of 12 recombinantly expressed UDP-glucuronosyltransferase enzymes with two different concentrations of the four selected substrates were performed. Highest levels of product were found only in incubations with the UGT1A3 isozyme proving the importance of this enzyme in the glucuronidation/ lactonization of these substrates.

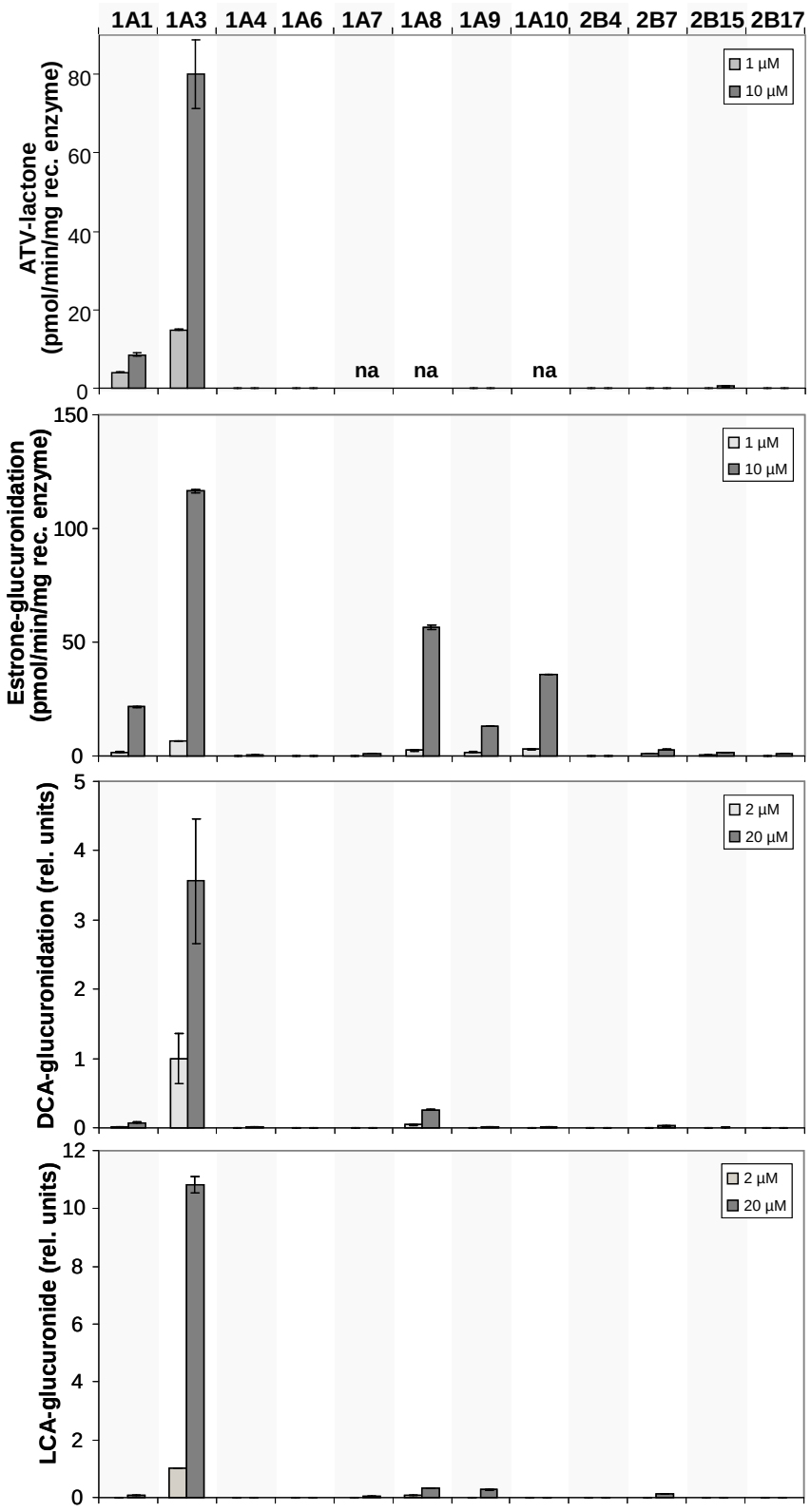


Figure 15: Atorvastatin-lactone, as well as, Estrone-3- and DCA-/LCA-24-glucuronide formation in UGT-isozymes. Substrate concentrations of 1 and 10µM (atorvastatin and estrone) or 2 and 20µM (DCA, LCA) were used in incubations with 25µg of recombinantly expressed UGT1A and UGT2B isozymes. Bars represent the mean of two incubation experiments and standard deviation is marked by error bars.

As presented in figure 15 above, atorvastatin-lactone was formed by UGT1A1and UGT1A3. The activity of UGT1A3 was 9.3- or 3.7-fold higher at the concentrations 10µM and 1µM respectively, than in the UGT1A1 isozyme. Detectable glucuronidation of estrone by other UGTs than UGT1A3 was found in all tested UGTs except UGT1A6 and 2B4. Significant amounts could be detected in UGT1A1, 1A8, 1A9 and 1A10 being 5.4- (3.8-), 2.1- (2.7-), 8.9- (3.7-) and 3.2- (2.3-) fold lower than UGT1A3 at 10µM (1µM), respectively.

Glucuronidation of DCA and LCA was found to be catalyzed by UGT1A3 in a highly specific manner. Only minor amounts of glucuronidated product were detected by incubations with the isozymes 1A1 and 1A8 in DCA, which had a 14- (22-) or 48- (74-) fold lower activity than UGT1A3 at 20µM (2µM). LCA-glucuronide formation was also catalyzed by 1A8, 1A9 and 2B7 having a 34- (12-), 40- (n.d.) or 93- (n.d)-fold lower glucuronidation than UGT1A3.

2.2.5.2.2 Biotransformation of atorvastatin, estrone, DCA and LCA in UGT1A3-haplotypes

Analyses performed in **section 2.2.5.1** had shown that the *UGT1A*- haplotype is not only a factor determining UGT1A3 expression, but also, via this increased expression, the cause for changed conjugating activity. Therefore, human liver microsomes were selected according to *UGT1A3*-haplotype (**1* and **2* homozygous and **2*, **3* and **6* heterozygous samples) and their activity for the four probe substrates was compared (figure 16).

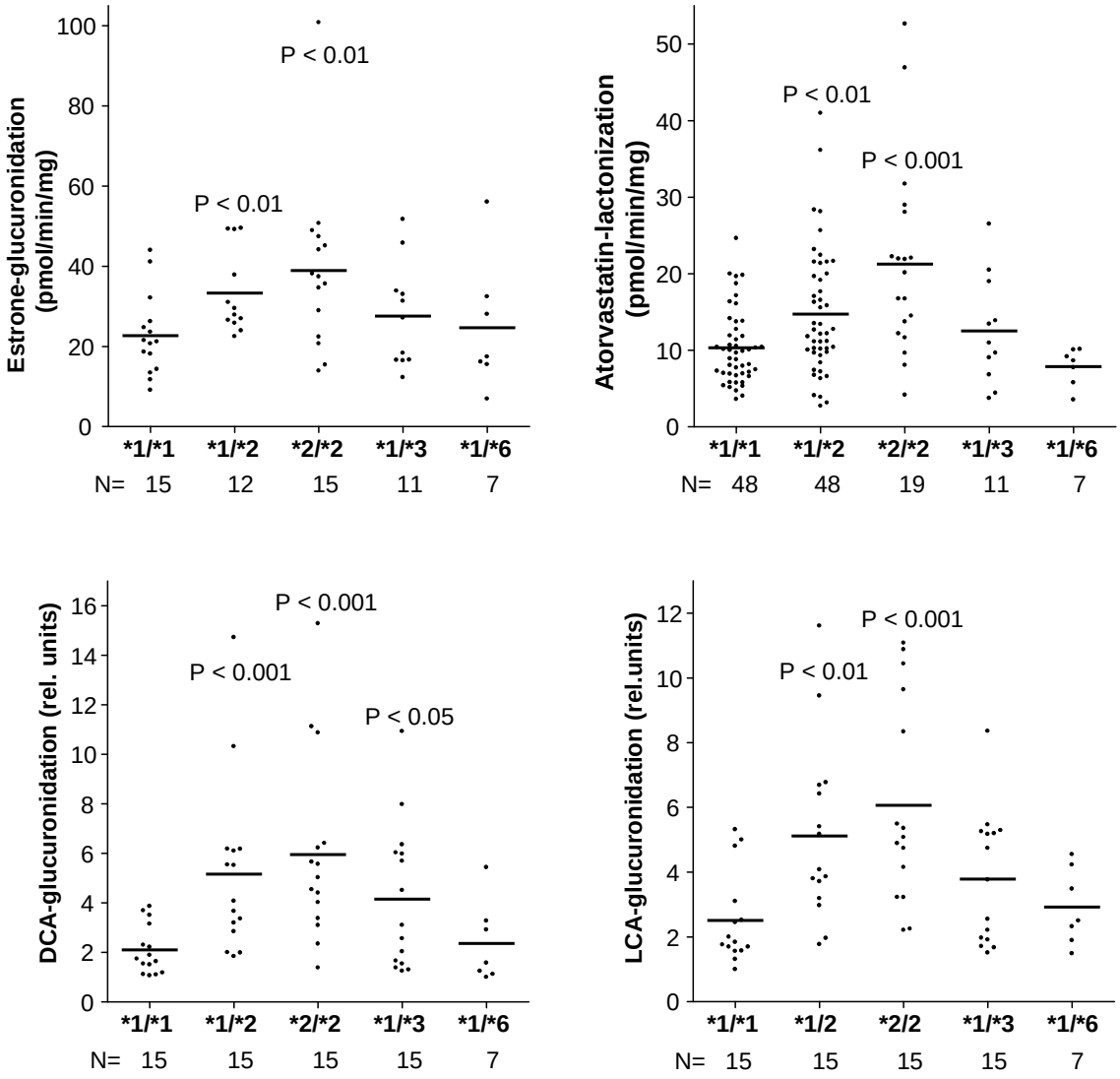


Figure 16: Glucuronidation/lactonization activity of human liver microsomes sorted for UGT1A3-haplotypes in probe substrates. Atorvastatin-lactone, as well as, Estrone-3- and DCA-/LCA-24-glucuronide formation was determined by incubations with 10µM or 20 µM of substrate in human liver microsomes. Plots indicate the median (horizontal line) and statistical significance as compared with wild-type (*1/*1) is indicated.

Homozygous followed by heterozygous carriers of the UGT1A3*2 haplotype were found to have the highest enzyme activity compared to wild-types (**1/*1*) in all tested substrates. Estrone-glucuronidation was 1.5- and 1.7-fold, atorvastatin-lactonization 1.4- and 2.1-fold, DCA 2.5- and 2.8-fold and LCA 2.0- and 2.4-fold higher in **2* heterozygous and homozygous compared to wild-type samples. Whereas **1/*6* showed identical activity as **1/*1* samples, carriers of the **3* haplotype had (non-significantly) increased activity in estrone (1.2-fold) , atorvastatin (1.2-fold) and LCV (1.5-fold), and a 2.0-fold significantly increased activity in DCA determinations.

When applying a **2* allele dependent linear model, the multiplicative effect of this allele was smallest in estrone (1.29-fold; 95%CI=1.10 to 1.51; P<0.01) and slightly higher in atorvastatin (1.38-fold; 95%CI=1.20 to 1.59; P<0.001). Highest allele dependent effects could be observed in LCA (1.55-fold; 95%CI=1.27 to 1.89; P<0.001) followed by DCA (1.62-fold; 95%CI=1.31 to 2.01; P<0.001), reflecting the high specificity of UGT1A3 for these substrates observed in the incubations with different UGT isozymes.

2.2.5.3 *UGT1A3*-haplotypes **1* and **2* in a recombinant transient expression system

Work presented in the previous section has shown the importance of the *UGT1A3** -haplotype for UGT1A3 expression and also for atorvastatin-lactonization, estrone- and bile-acid-glucuronidation. Analyses to define how *UGT1A3*2* changes the expression and the acivity of UGT1A3 and were therefore the focus of the following experiments.

*2.2.5.3.1 Influence of UGT1A3*2 on enzyme expression*

Although other SNPs linked to *UGT1A3*2* have to be taken into consideration, exonic polymorphisms seemed likely candidates for increased expression. To gain further evidence for their role, a recombinant transient expression system was used to compare the expression levels of *UGT1A3*1* and *UGT1A3*2*. Full length wild-type and mutant UGT1A3 (including 66 bases 5' and 22 bases 3'UTR) were cloned from genotyped human liver cDNA into an expression plasmid under the control of a CMV promoter and transiently expressed in COS cells (pSRM16 (*UGT1A3*1*) and pSRM14 (*UGT1A3*2*)). Preliminary expression experiments using β-Galactosidase co-transfection were used to show similar transfection efficiencies of *UGT1A3*1* and *UGT1A3*2* in cell-lysate (data not shown). Expression was scaled up under similar conditions and microsomes were prepared expressing both UGT1A3 variants. Microsomal preparations of UGT1A3**1* and UGT1A3**2* were analyzed by immunoblotting (figure 17).

A comparison of the expression levels showed that microsomal preparations from recombinant *UGT1A3*1* was 0.8-fold and **2* was 6.3-fold the expression detected in pooled human liver microsomes at the same concentration. In a direct match *UGT1A3*2* is 7.7-fold higher expressed than *UGT1A3*1* reflecting the mean 7.3-fold higher expression level of homozygous **1* compared to **2* carriers in the Caucasian population. Hence, the increased expression of *UGT1A3*2* was found to be clearly resulting from the exonic polymorphisms.

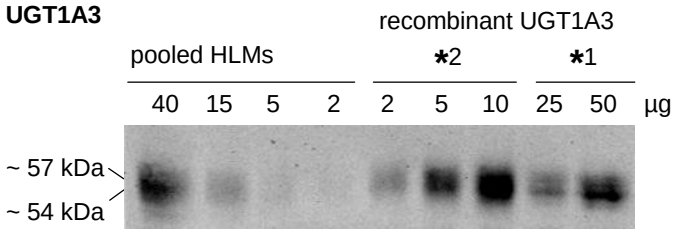


Figure 17: Comparison of expression levels of recombinantly expressed *UGT1A3*1*, *UGT1A3*2* and UGT1A3 in pooled human liver microsomes. Microsomal fractions were prepared from COS1 cells expressing recombinant UGT1A3 protein. Immunoblots were stained with specific antibodies against UGT1A3. Intensities were compared using the ODYSSEY infrared imaging system (LICOR).

*2.2.5.3.2 Influence of UGT1A3*2 on specific activity*

UGT1A3**2* differs in two amino acids, *W11R* and *V47A*, from *UGT1A3*1* enzymes. These changes might affect the specific activity of UGT1A3. To determine the influence of the **2* haplotype on specific activity, transiently expressed wild-type (**1*) and **2* UGT1A3 as described above were used for the preparation of microsomes and used for activity determinations. For comparing the two haplotype variants, activity was determined at 20µM. This concentration was chosen, because determinations of kinetics had sown K_m values of LCA and DCA close to this concentration (LCA: K_m=15µM and DCA: K_m=23µM). So, comparisons of specific activity were done within the linear range of the substrate concentration. Interestingly, a 5.8-fold or 5.5-fold reduced specific activity of *UGT1A3*2* could be observed when compared to the specific activity of the wild-type in LCA- or DCA-glucuronidation, respectively.

2.3. Atorvastatin-lactone hydrolysis

Hydrolysis of statin-lactones occurs spontaneously and enzymatically (Vickers et al., 1990, Billecke et al., 2000). Previous reports have shown an enzymatic conversion for simvastatin and lovastatin, two statins, which are administered as lactone pro-drugs. Simvastatin-lactone hydrolysis was determined in blood serum, whereas recombinantly expressed paraoxonases were used to analyze lovastatin-lactone hydrolysis (Draganov et al., 2005, Suchocka et al., 2006).

2.3.1 General role of enzymatic lactone hydrolysis

Preliminary experiments based on results from a study on the hydrolysis of lovastatin-lactone indicated hydrolysis of lovastatin- and atorvastatin-lactone in human liver microsomes. A comparison of enzymatic compared to spontaneous conversion, the latter identified by identical incubations with heat-inactivated protein fractions, revealed enzymatic conversion to be 1.3-fold higher for lovastatin-lactone and 3.8-fold higher for atorvastatin-lactone in microsomal fractions (data not shown). Control incubations with heat- inactivated protein were included and used for subtracting background in all subsequent analyses with atorvastatin-lactone.

2.3.2. Variability of microsomal atorvastatin-lactone-hydrolysis

Following this, enzymatic atorvastatin-lactone hydrolysis was detected in a large number of microsomal fractions (N=142 of N=150). While establishing the atorvastatin-lactone hydrolysis assay, we could show that the activity could be increased by a mean value of 20% in incubations containing 1mM Ca^{2+}, a fact, which was used in subsequent analyses to increase enzymatic activity, although these reactions generally are not in need of co-substrates. Incubation time, substrate concentration and protein content were chosen with care to guarantee measurements within the linear range for each condition. Remarkably, optimized conditions allowed using a low protein content of 5µg per incubation.

Variability of the formation of atorvastatin-lactone in the cohort of human liver samples is presented in figure 18. Enzymatic lactone hydrolysis ranged between 0 to 42% of total atorvastatin-acid formation, emphasizing an important role of this reaction in the interconversion of lactone to acid. Comparing the coefficients of variation, UDP-glucuronosyltransferase 1A3 dependant lactone formation (see figure 11) and enzymatic lactone hydrolysis are comparably variable (62% and 54%, respectively). It should be noted

that an influence of UDP-glucuronosyltransferases on lactone hydrolysis could be excluded as no co-substrate (UDP-glucuronic acid) was present in these reactions.

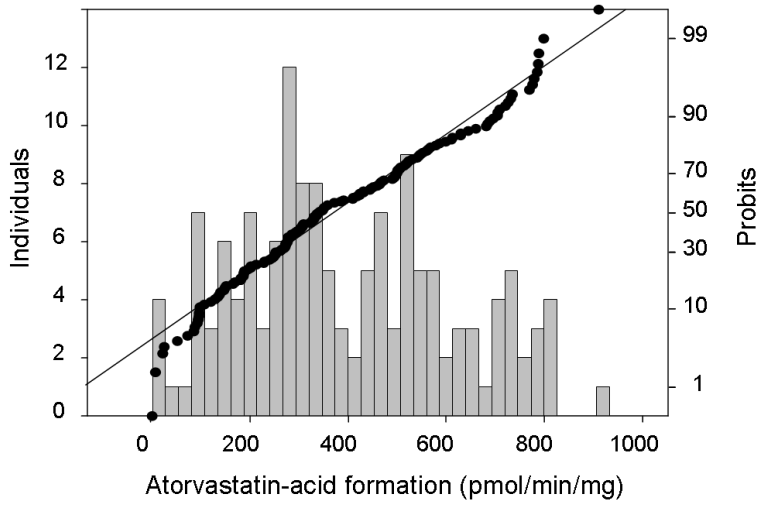


Figure 18: Frequency histogram (left axis) and cumulated frequency plot (right axis) showing population distribution of the hydrolysis of atorvastatin-lactone (10µM) to atorvastatin-acid in human liver microsomes (N=142). Incubations (5 µg of microsomal protein) were performed at 37°C for 30 minutes in the presence of 1mM CaCl. Atorvastatin acid was quantitated by LC-MS/MS analysis.

2.3.3. Screening of candidate esterases

Next, this study aimed to identify the enzyme(s) responsible for this conversion. Esterase activity has been described for several enzymes, possibly involved in the hydrolysis of lactones (Testa and Mayer, 2003). To identify candidates, I took advantage of existing genome-wide expression data available for the human liver cohort. Table 6 shows the result of a correlation analysis between atorvastatin-lactone hydrolysis and mRNA expression data (N=150) of candidate esterases acetylcholinesterase (ACHE), acyl-CoA thioesterase 1 (ACOT1), albumin (ALB), butyrylcholinesterase (BCHE), carboxyl ester lipase (CEL), carboxylesterase (CES1,2,3,4,7), paraoxonases (PON1,2,3), lysosomal acid, cholesterol esterase (LIPA), sialic acid acetylesterase (SIAE). Interestingly, the two enzymes with the highest correlation coefficients, PON1 and PON3, have been reported to be involved in simvastatin- and lovastatin-lactone hydrolysis (Draganov et al., 2005; Suchocka et al., 2006). Additional positive correlations were observed for BCHE and CES3, as well as, negative correlations for PON2 and LIPA.

Table 6: Correlation analysis of candidate gene expression and atorvastatin-lactone hydrolysis.

	ACHE	ACOT1	ALB	BCHE	CEL	CES1	CES2	CES3
atorvastatin-acid formation	* $r_s = 0.21$	n.s.	n.s.	*** $r_s = 0.38$	n.s.	* $r_s = 0.24$	n.s.	*** $r_s = 0.35$
	CES4	**CES7**	**PON1**	**PON2**	**PON3**	**LIPA**	**SIAE**	
atorvastatin-acid formation	n.s.	n.s.	*** $r_s = 0.40$	*** $r_s = -0.39$	*** $r_s = 0.52$	*** $r_s = -0.32$	** $r_s = -0.27$	

2.3.4 Population variability of microsomal PON1 and PON3 expression

Further experiments focused on PON1 and PON3 as their mRNA expression showed the strongest correlation to atorvastatin-lactone hydrolysis.

2.3.4.1 mRNA expression analyses of PON1 and PON3

In an approach to quantify distinct mRNA transcripts of PON1 (four splice variants; Ensembl: ENSG00000105852) and PON3 (9 splice variants; Ensembl: ENSG00000005421) by real-time PCR, preliminary amplifications identified the predicted PON1 and PON3 *wild-type* transcripts and a novel variant PON3 mRNA species. This *PON3 splice-variant* was shown by sequence analysis to contain an additional exon of 144bp (translating to 48 amino acids) between exon 3 and exon 4 (figure 19). No other variants were detected using primers for full length mRNA amplification in a subset of 20 samples. Comparing the average expression levels, the *wild-type PON3* transcript was 3.1-fold higher expressed than the *PON3 splice-variant*. Realtime-quantification of mRNA expression of PON1 and PON3 revealed that *PON3 wild-type* is on average 5.1-fold higher expressed than *PON1* mRNA (for correlations see 2.3.5).

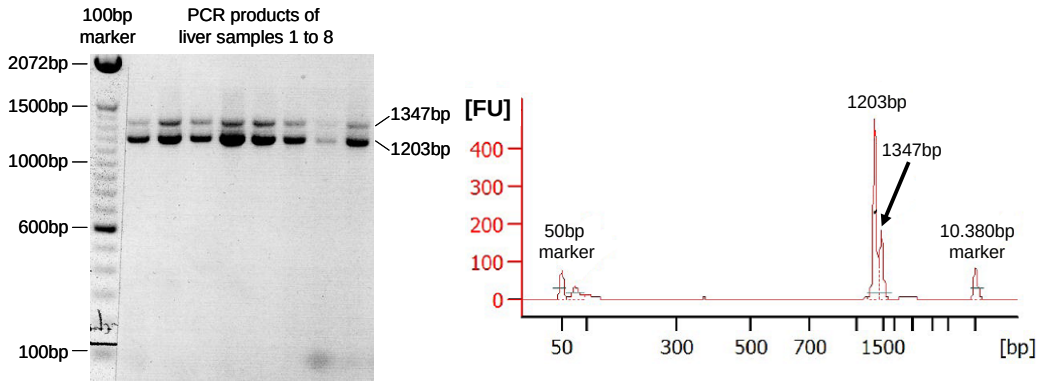


Figure 19: *PON3* gene PCR products amplified from cDNA of liver samples. *PON3 wild-type* (1203bp) and *PON3 splice-variant* (1347bp) bands or peaks analyzed by agarose gel electrophoresis (left, liver samples 1 to 8) and Agilent 2100 bioanalyzer DNA electropherogram (right, 1 exemplary sample). PCR products of 20 liver cDNAs were amplified via PCR with primers PON3_komplett_f and PON3_komplett_r (table 13).

2.3.4.2 Protein expression of PON1 and PON3

Protein expression of PON1 and PON3 in human liver microsomes was quantified relatively by Western blot analysis (figure 20) using monoclonal antibodies. PON1 was identified as a triple band in a range corresponding to 35 to 40kDa matching literature data from purified human serum paraoxonase (Furlong et al., 1991, Gan et al., 1991). PON3, in contrast, was found as a single band at 38kDa in human liver microsomes. In cytosolic samples and also in total cell-lysate from primary human hepatocytes two bands at 21 and 35kDa could be identified, but not the microsomal band at 38kDa. The PON3 *variant* was not detected.

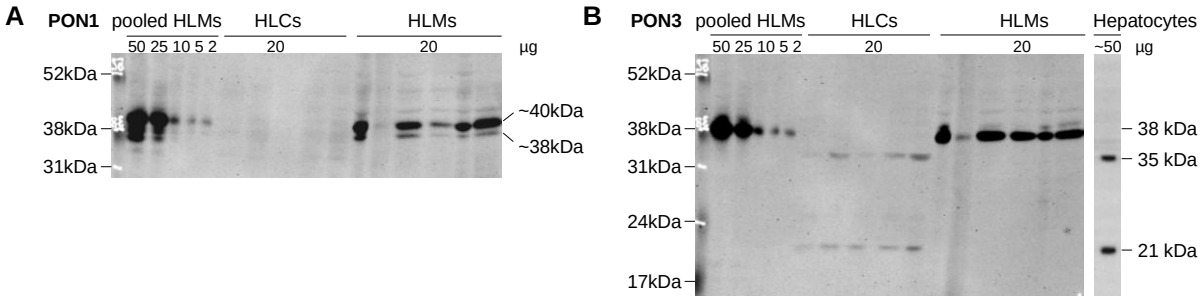


Figure 20: Immunoblots of human liver cytosole (HLCs) and microsomes (HLMs) of livers 1 to 5 stained with specific antibodies against (A) PON1 and (B) PON3 (performed on identical blot after stripping). Analysis was performed on cytosolic and microsomal fractions of livers 1 to 5, on a human liver microsome pool (lane 11) and on lysate of primary human hepatocytes. The PON3 blot of a primary human hepatocyte sample was performed by J.K. Rieger on a LI-COR MPX™ (Multiplex) Blotter.

Since the various immune-reactive bands suggested both enzymes to be glycosylated, endoglycosidase treatment was performed (figure 21). Remarkably, unglycosylated PON1 and PON3 were shifted towards a single 35kDa band with both antibodies suggesting both

proteins to be highly glycosylated in microsomes. This band corresponded to one of the two bands detected in human liver cytosole and in hepatocytes. The observed microsomal pattern of three bands for PON1 and one band for PON3 corresponded to three reported glycosylation sites (amino acids N226, N252 and N323) on PON1 and one site (N323) on PON3 (Liu et al., 2005). For quantification, the liver samples were analyzed in their glycosylated state and the relevant signals were combined.

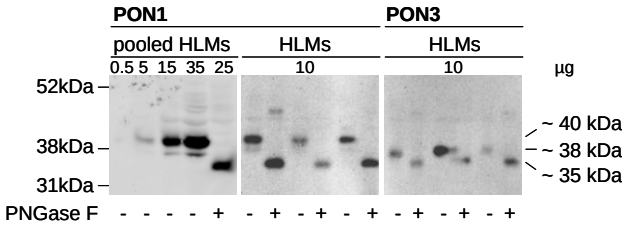


Figure 21: Immunoblots of human liver microsomes (HLMs) to analyse the effect of deglycosylation on PON1 and PON3 electrophoretic mobility. Samples were pre-treated with (+) or without (−) endoglycosidase PNGase F.

Final analysis revealed that both PON1 and PON3 varied considerably within the analyzed phenotypes of expression and atorvastatin-lactone hydrolysis. Table 7 shows a comparison of variability parameters at mRNA and protein/activity data. All phenotypes were not normally distributed in this cohort. Comparing the coefficients of variation, protein expression of PON1 and PON3 had similar values of 69% and 74%. Atorvastatin-lactone hydrolysis variation was lower (54%). Whereas *PON3 wild-type* and *splice-variant* mRNA variation was lowest (40% and 43% respectively), PON1 mRNA variation was highest (150%) resulting mainly from few samples with very high expression.

Table 7: Population variability of hepatic PON1 and PON3 expression phenotypes.

	PON1		PON3			Atorvastatin-acid formation (pmol/ min/mg)
	mRNA/ RPLP0 relative units	Protein (µg/mg)	*wild-type* mRNA/ RPLP0 relative units	*variant* mRNA/ RPLP0 relative units	Protein (µg/mg)	
Minimum	0.61	51.40	7.29	2.20	0.90	6.67
Median	5.84	620.0	28.73	9.50	14.10	309.70
Maximum	120.30	2200	73.74	22.51	74.65	816.0
Ratio max./min.	196.25	42.80	10.12	10.23	82.94	122.34
Normal distribution	No	No	No	No	No	No
Sample skewness	6.88	1.05	0.67	0.58	1.44	0.30
Coefficient of variation (%)	150	69	40	43	74	54

2.3.5 Identification of responsible hydrolysing enzymes by correlation analyses

To confirm a role of PON1 and PON3, correlation analyses were performed on the population data. As shown in table 8, stronger associations were observed for three phenotype pairs. Remarkably, atorvastatin-lactone hydrolysis correlated with similarly high Spearman coefficients to PON1 and PON3 protein, indicating their comparable contribution to this reaction in microsomes. Also, the mRNAs of PON1 and PON3 (*wild-type* and *splice-variant*) were significantly correlated with activity data, although with lower Spearman coefficients. In addition correlations were observed between transcripts, as well as, between PON1 and PON3 protein. As no noticeable cross-staining was detected for both antibodies (see figures 20 and 21), this might point to a certain extent of co-regulation of PON1 and PON3.

Table 8: Correlation analysis of PON1 and PON3 (WT, *wild-type*; VAR, *splice-variant*) expression and atorvastatin-acid formation. Significant associations above r_s=0.45 are marked in grey.

PON1 protein	PON3 WT mRNA (exon 3-4)	PON3 VAR mRNA (exon 3a-4)	PON3 protein	atorvastatin-acid formation	
*** r_s = 0.45	*** r_s = 0.42	*** r_s = 0.40	n.s.	*** r_s = 0.28	**PON1 mRNA** (exon 8-9)
	*** r_s = 0.27	*** r_s = 0.28	*** r_s = 0.48	*** r_s = 0.60	**PON1 protein**
		*** r_s = 0.90	*** r_s = 0.31	*** r_s = 0.33	**PON3 WT mRNA** (exon 3-4)
			*** r_s = 0.32	*** r_s = 0.32	**PON3 VAR mRNA** (exon 3a-4)
				*** r_s = 0.62	**PON3 protein**

2.3.6 Genetic variability of the *PON*-locus in a Caucasian population

Genetic variability within the *PON*-locus was analyzed by selecting known functional SNPs, which cover the coding and regulatory regions of the two candidate paraoxonases, as well as, tagging SNPs for the less well characterized *PON3* gene. Therefore, genotyping included seven *PON3* promoter SNPs, 11 coding SNPs, 14 intronic SNPs and one 3'UTR SNP. Additionally, three *PON1* promoter SNPs, two coding SNPs and two intergenic SNPs between *PON3* and *PON1* were included. Selection criteria are described in detail in Methods 4.7.1.

A summary of the selected polymorphisms and the genotyping results are shown in table 9. Frequencies of the variant allele (VAF) are presented in comparison to database or literature VAF. Major frequency deviations were only observed in comparisons to SeattleSNP database, probably because only 23 subjects had been analyzed in this database. SNP19 was much more common in this Caucasian population than in the data from a Chinese population (Wang et al., 2003). Deviation from Hardy Weinberg equilibrium was tested for all SNPs but was not detected. Genomic position was given according to reference sequence of *PON3* NG_008726.1 modified by adding *PON1* NG_008779 and the intergenic region in between. SNP positions in modified regions were marked by brackets.

Table 9: Characteristics and variant allele frequencies (VAF) of SNPs determined in 150 Caucasian samples.

SNP	*Gene*	*SNP ID dbSNP or Seattle SNP*	*Genomic Position*	*Base Change*	*Residue Change*	*Region*	*VAF*	
							IKP liver bank	*Database; Literature*
1	PON3	var1496	42	A/G		promoter	0.295	0.200
2	PON3	var2115	661	A/T		promoter	0.087	0.200
3	PON3	var2375	921	G/A		promoter	0.288	0.200
4	PON3	rs11767787	3935	A/G		promoter	0.309	0.241
5	PON3	rs17885453	4232	C/T		promoter	0.014	0.023
6	PON3	rs17882539	4280	C/T		promoter	0.295	0.181
7	PON3	rs2072200	4528	C/G		promoter	0.198	0.272
8	PON3	rs13226149	5088	C/T	F21	exonic	0.309	0.250
9	PON3	var9827	8372	G/A		intronic	0.183	0.300
10	PON3	rs10487132	10383	T/C		intronic	0.360	0.383
11	PON3	var12788	11333	C/T		intronic	0.486	0.430
12	PON3	rs1003504	11895	A/G		intronic	0.018	0.022
13	PON3	rs978903	26521	T/C		intronic	0.497	0.458
14	PON3	Campo219	29055	C/G	G51	intronic	0.004	0.007
15	PON3	rs1053275	29133	G/A	A99	exonic	0.493	0.466
16	PON3	rs2375003	29155	G/A	D107N	exonic	0	0
17	PON3	rs2375002	29330	T/A		intronic	0.09	0.043
18	PON3	rs468	32735	T/C		intronic	0.096	0.025
19	PON3	Wang.133	33772	G/T		intronic	0.004	0.227
20	PON3	rs3757708	33775	A/C		intronic	0.489	0.483
21	PON3	rs17879114	33898	G/A	V126	exonic	0	0
22	PON3	rs17878827	33956	G/A	E146K	exonic	0	0
23	PON3	var37120	35664	G/A		intronic	0.036	0.110
24	PON3	rs9640632	36134	A/G		intronic	0.482	0.483
25	PON3	rs17883013	37354	C/A	A179D	exonic	0	0
26	PON3	rs17880470	37427	T/C	Y203	exonic	0.014	0.022
27	PON3	Ranade	38537	A/G	Y233C	exonic	0	0
28	PON3	var40512	39056	A/G		intronic	0.183	0.300
29	PON3	rs2057682	39924	C/G		intronic	0.142	0.076
30	PON3	rs7778771	40352	C/T		intronic	0.014	0.025
31	PON3	Campo931	41269	T/A	S311T	exonic	0	0.002
32	PON3	Campo971	41309	G/A	G324D	exonic	0	0.006
33	PON3	rs17885558	41438	C/T		3'UTR	0.014	0.025
34		var45486	(44028)	G/T		intergenic	0.014	0.050
35		var55146	(53688)	G/A		intergenic	0.050	0.100
36	PON1	rs757158	(75164)	G/A		promoter	0.486	0.408
37	PON1	rs854571	(76073)	A/G		promoter	0.285	0.267
38	PON1	rs705379	(76797)	T/C		promoter	0.460	0.389
39	PON1	rs854560	(84608)	T/A	L55M	exonic	0.335	0.397
40	PON1	rs662	(93246)	A/G	Q192R	exonic	0.352	0.358

The calculation of pairwise linkage disequilibrium (LD) values of the *PON*-locus revealed a block of tightly linked polymorphisms comprising the *PON3* promoter region, several intronic polymorphisms and the non-synonymous polymorphisms F21 (rs13226149) and A99 (rs1053275; D'=0.88-1.0; r^2=0.04-1; figure 22). Of interest, this block is also linked to the *-1741G>A PON1* promoter polymorphism (D'=0.49-1.0; r^2=0.09-0.43). Moreover, linkage was identified between *PON1* promoter polymorphisms and L55M (D'=0.59-0.87; r^2=0.14-0.19). *PON1* Q192R on the other hand was only linked to L55M (D'=0.95; r^2=0.23), but neither to *PON1* promoter polymorphisms nor to polymorphisms of the *PON3* linkage block. Linkage was additionally detected among the rare polymorphisms *PON3-794C>T, 3´UTR* rs17885558 and the intergenic polymorphism rs7778771 (VAF=1.3%; (D'=1.0; r^2=1.0).

Figure 22: Pairwise linkage disequilibrium of the *PON*-locus. The map was generated using Haploview 4.2 from data related to 40 SNPs detected in livers from the Caucasian population. D'=1: bright red (LOD>2) or blue (LOD<2); D'<1: shades of pink (LOD>2) or white (LOD<2).

2.3.7 Association of PON-polymorphisms with PON-phenotypes

Univariate analysis was carried out for association between single SNP genotypes and the PON phenotypes, atorvastatin-lactone hydrolysis, PON1 and PON3 protein expression (figures 23-25).

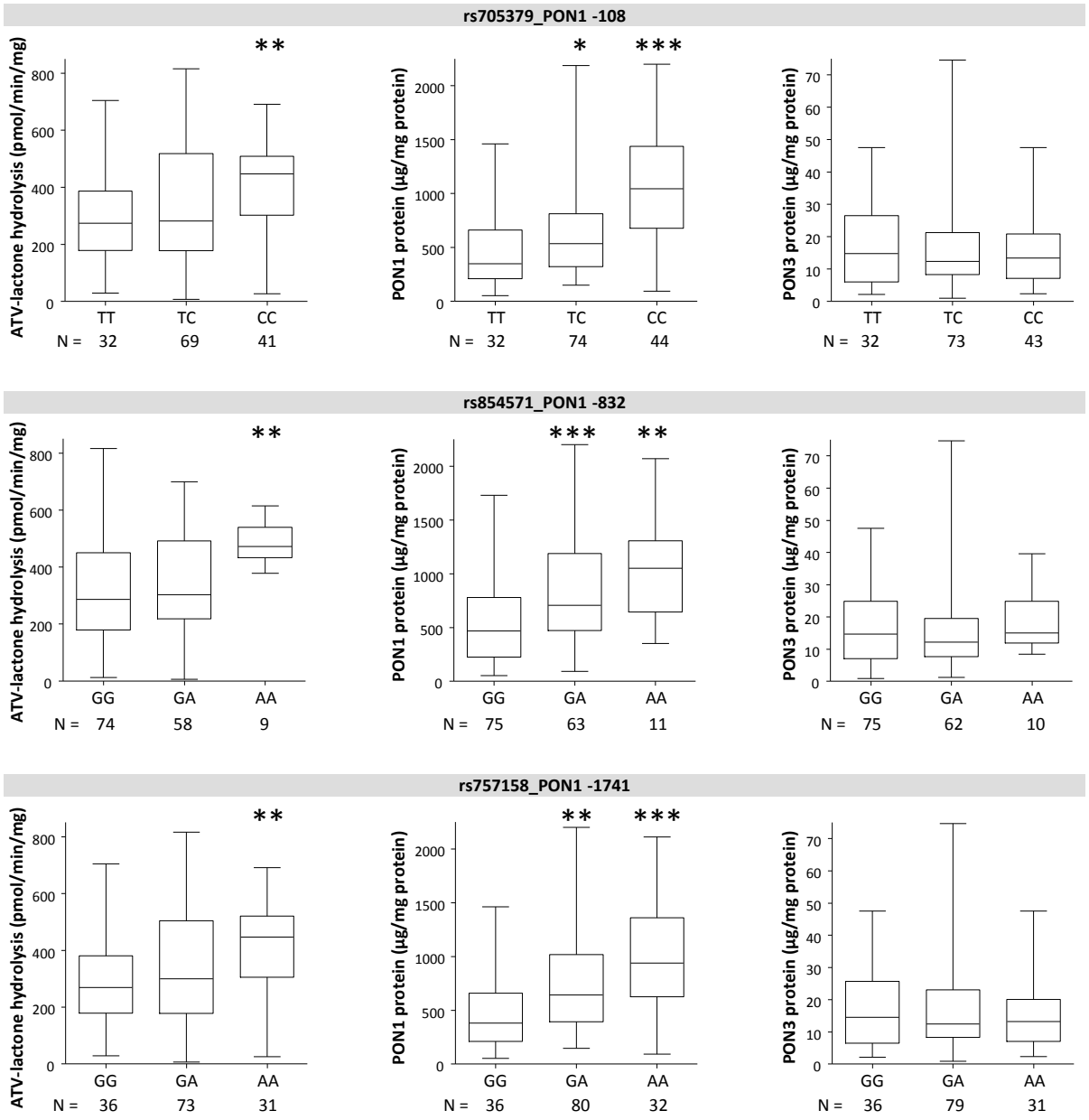


Figure 23: Boxplots of PON1 and PON3 protein expression and microsomal atorvastatin-lactone hydrolysis in liver microsomes for indicated polymorphisms. Heterozygotes and homozygotes of variant allele are compared with homozygotes of reference allele by Wilcoxon-Mann-Whitney-Tests Significance levels are indicated for P<0.05 (*), P<0.01 (**) and P<0.001 (***).

PON1 promoter polymorphisms *-108T>C*, *-832G>A*, and *-1741G>A* were associated with significantly increased atorvastatin-lactone hydrolysis (up to 1.1-fold increased median values in heterozygous and 1.6- to 1.7-fold in homozygous carriers, respectively) and are, as shown

above (figure 22) highly linked. These changes corresponded to increased PON1 protein (1.5- to 1.7-fold higher in heterozygous and 2.3- to 3.0-fold in homozygous samples, respectively; tested by Wilcoxon-Mann-Whitney, not adjusted for multiple testing). PON3 protein, on the other hand, was unchanged in these variants. Considering the mRNA transcripts, similar effects were seen for PON1 (data not shown).

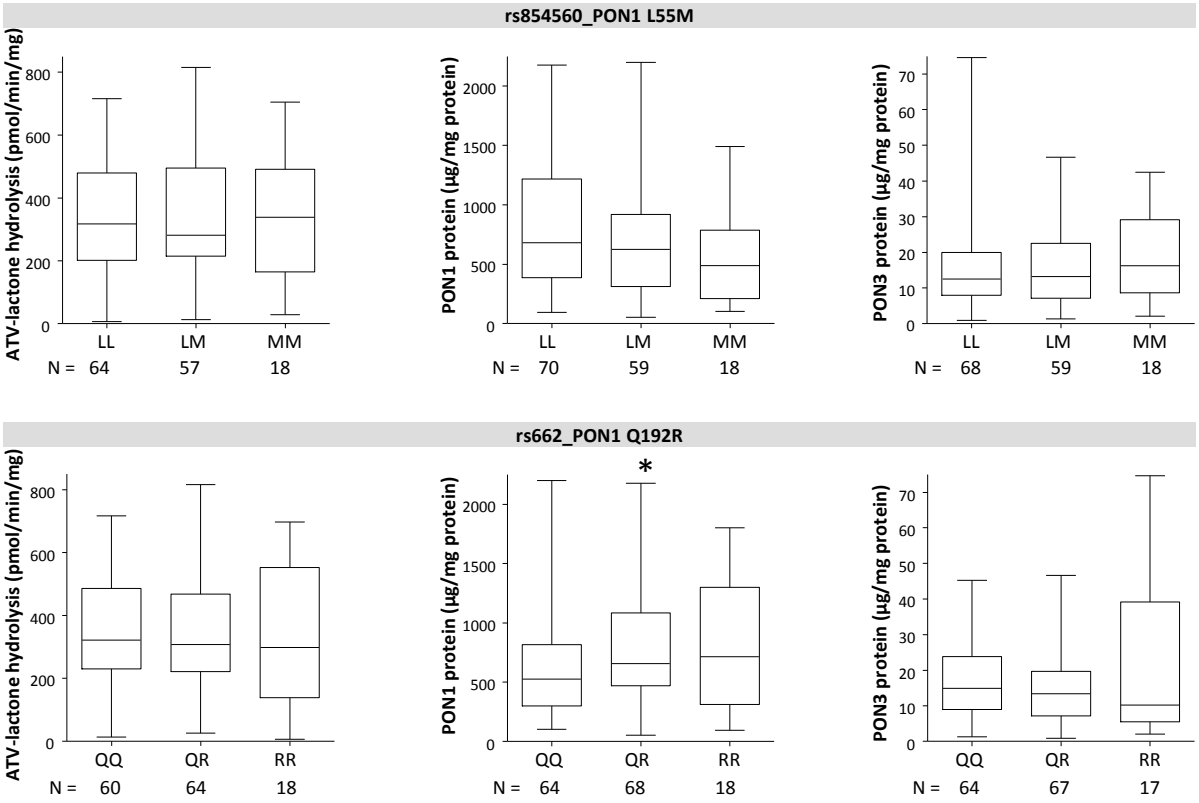


Figure 24: Boxplots of PON1 and PON3 protein expression and microsomal atorvastatin-lactone hydrolysis in liver microsomes for indicated polymorphisms. Heterozygotes and homozygotes of variant allele are compared with homozygotes of reference allele by Wilcoxon-Mann-Whitney-Tests Significance levels are indicated for P<0.05 (*), P<0.01 (**) and P<0.001 (***).

L55M and Q192R, two amino acid changes in PON1 were previously described to affect the kinetics of various substrates (Costa and Furlong, 2002). Both were not consistently associated with changes in either atorvastatin-lactone hydrolysis or PON1/PON3 protein expression, although there was a trend for decreased PON1 expression in relation to L55M and a trend for increased PON1 expression in relation to Q192R (figure 24).

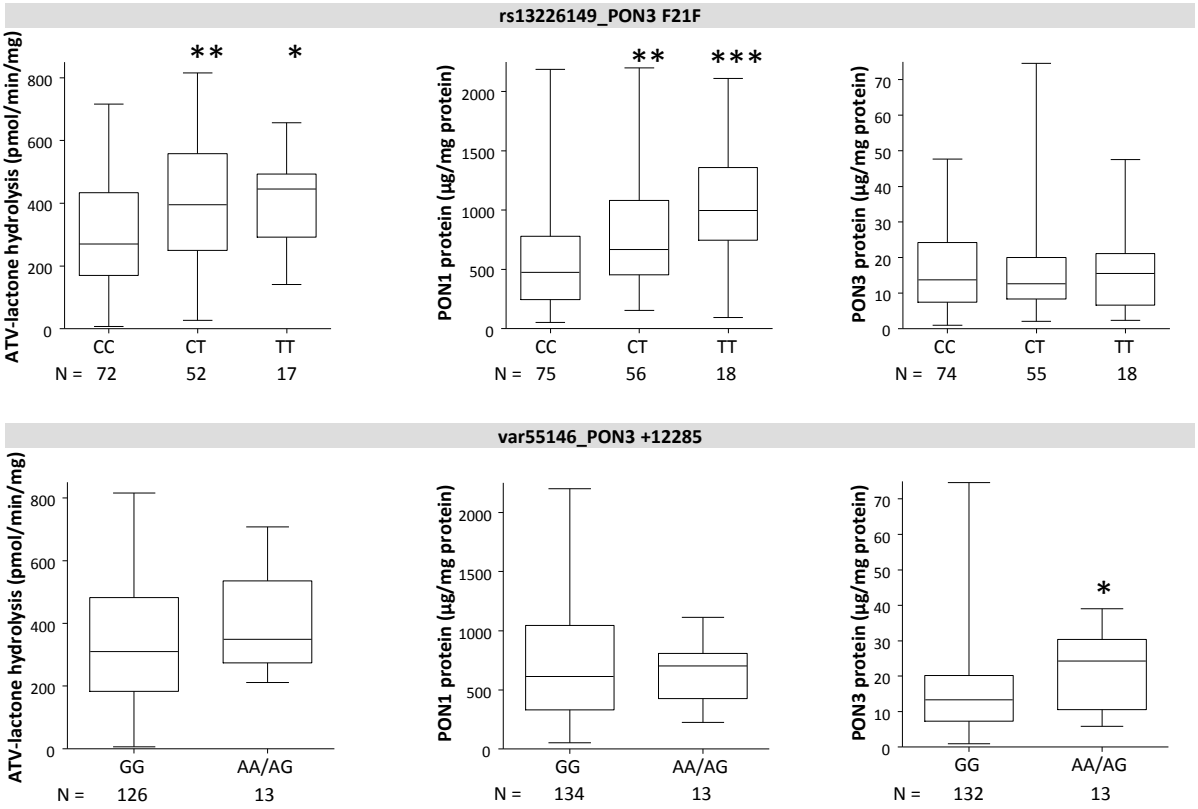


Figure 25: Boxplots of PON1 and PON3 protein expression and microsomal atorvastatin-lactone hydrolysis in liver microsomes for indicated polymorphisms. Heterozygotes and homozygotes of variant allele are compared with homozygotes of reference allele by Wilcoxon-Mann-Whitney-Tests Significance levels are indicated for P<0.05 (*), P<0.01 (**) and P<0.001 (***).

In a linkage analysis of PON3 SNPs, F21 (rs13226149) was identified as a suitable marker SNP for a haplotype block comprising several PON3 promoter polymorphisms, A99 (rs1053275), and several intronic variations (figure 22). F21 was associated with increased atorvastatin-lactone hydrolysis and with increased PON1 protein expression, but unchanged PON3 expression (figure 25).

In contrast to the above SNPs, which were only associated with PON1 expression, the intergenic variation at +12285 (var55146), was associated with 1.8-fold increased PON3 protein but only minimally changed activity or PON1 protein. Because only one homozygote was found for this variant, homozygous and heterozygous carriers were analyzed together (figure 25).

2.3.8 Analysis of PON haplotypes and their association with PON-related phenotypes

SNP genotyping data made it possible to deduce haplotypes for the *PON*-locus. To limit the number of generated haplotypes this deduction only included SNPs with VAF<2% (see methods 4.9). Furthermore, because the above analysis revealed that PON3 polymorphisms are associated with changes in PON1 phenotype we defined the haplotypes such that they included both genes and the intergenic region. This approach resulted in the definition of 13 haplotypes with predicted frequencies between 0.022 and 0.206 (table 10). As it was the aim to classify functionally relevant haplotypes, the results from the univariate analyses were used to derive 7 major haplotypes. Thus, haplotypes **3* to **7* comprised the *PON3* linkage block including F21 and/or *PON1* promoter SNPs (figure 3). Haplotype **2* was defined by the intergenic variant +12285, and **1* comprised all haplotypes lacking these variants. Of note, definition of these haplotypes did not consider the amino acid variants L55M and Q192R, which had no apparent functional influence in this study.

Table 10: Structure of deduced *PON*-haplotypes.

Haplo-type ID	-4984	-4365	-4105	-1091	-746	-498	F21F	intron2	intron2	intron3	intron3	A99A	intron4	intron4	intron4	intron5	intron5	intron6	intron6	+12285	-1741	-832	-108	L55M	Q192R	Predicted Frequency	
PON*1a	A	A	G	A	C	C	C	G	C	C	T	G	A	T	A	G	A	A	C	G	G	G	T	A	A		0.206
PON*1b																								T	G		0.055
PON*1c		T				T		A	T	T	C	A			C		G	G						T			0.048
PON*1d		T				T		A	T	T	C	A			C		G	G						T	G		0.031
PON*1e						T		A	T	T	C	A			C	A	G	G						T	G	**0.368**	0.028
PON*2																				A						**0.032**	0.032
PON*3	G		A	G	T		T		T	T	C	A			C		G				A		C	T	G	**0.101**	0.101
PON*4a	G		A	G	T		T		T	T	C	A			C		G				A	A	C	T	G		0.044
PON*4b	G		A	G	T		T		T	T	C	A			C		G				A	A	C	T		**0.113**	0.069
PON*5a						T		A	T	T	C	A			C		G	G			A	A	C	T	G		0.028
PON*5b									T										G		A	A	C	T		**0.051**	0.022
PON*6																						A	C	T		**0.034**	0.034
PON*7									T				T	A					G		A		C	T		**0.076**	0.076

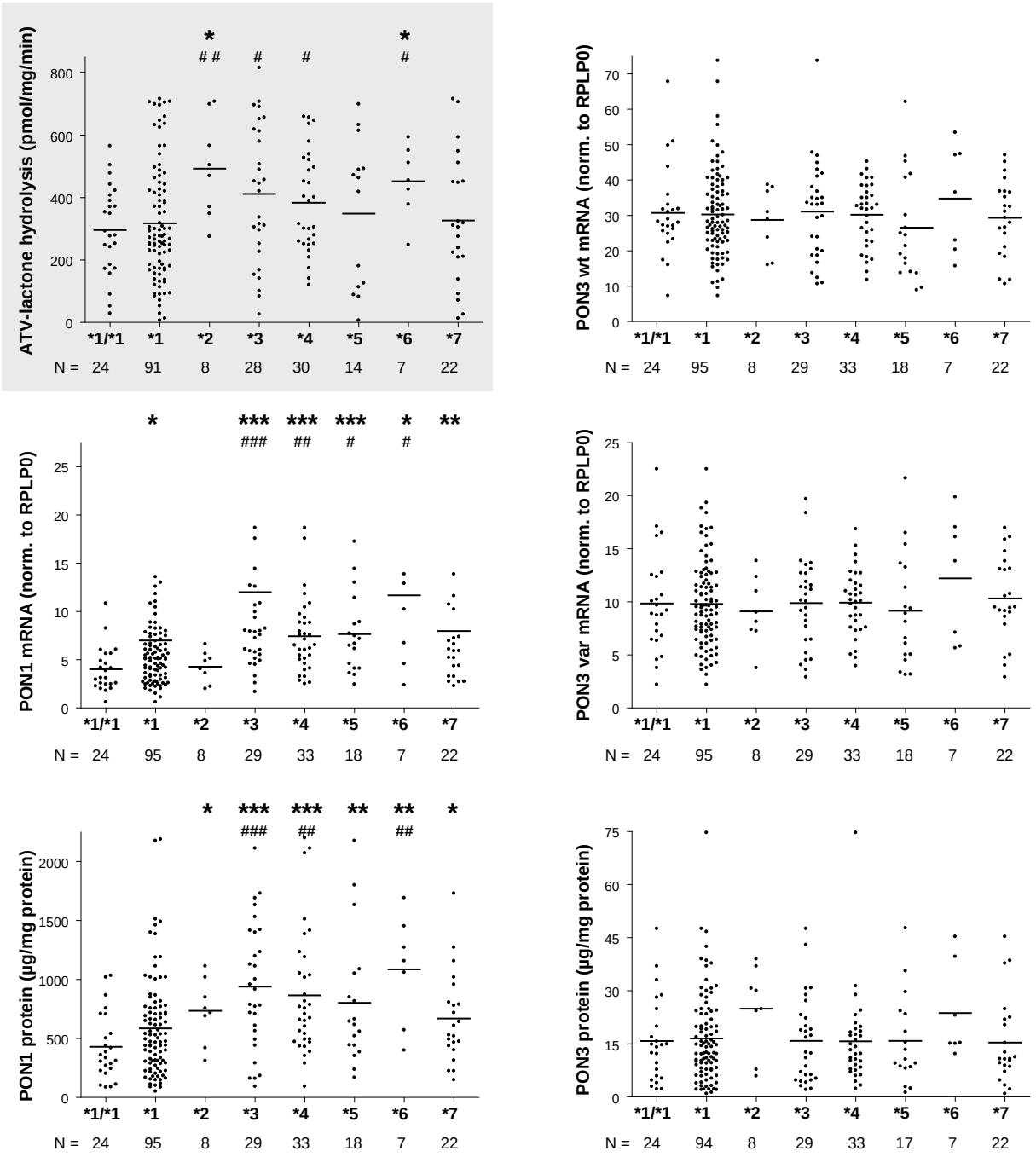


Figure 26: *PON*-locus haplotype-phenotype relationships in human liver. Atorvastatin-lactone hydrolysis, PON1 and PON3 mRNA and microsomal protein are displayed for the indicated haplotypes. Horizontal lines indicate the median. Wilcoxon-Mann-Whitney-Tests were applied to compare **2* to **7* carriers with **1/*1* (marked by *) or **1* carriers (marked by #). Significance levels not adjusted for multiple testing are indicated for P<0.05 (* or #), P<0.01 (** or ##) and P<0.001 (*** or ###). After adjusting for multiple testing **3, *4,*5,*7* against **1/*1* or **3* and**4* against **1* were significantly different on PON1 mRNA level and **2* to **7* against **1/*1* or **3*, **4* and **6* against **1* were significantly different on PON1 protein level.

In associations of these 7 haplotypes, atorvastatin-lactone hydrolysis was strikingly increased in carriers of haplotypes **2* (1.7-fold) and **6* (1.6-fold) as compared to homozygotes of haplotype **1* (figure 26). Less pronounced but still significant changes were also found between carriers of haplotypes **2, *3, *4* and **6* as compared to heterozygous carriers of **1*.

PON1 mRNA expression was increased ~2-fold in **3, *4,* *5, **7* and 2.7-fold in the **6* haplotype carriers but only marginally in the **2* haplotype. PON1 protein expression resembled the mRNA pattern with ~2.1- and 1.6-fold increased expression in **2* and **7,* respectively, ~2-fold increase in **3, *4,*5,* and 3.3-fold increase in **6*. For PON3 no significant associations were found with respect to these haplotypes, although we noted increased expression of PON3 protein in most carriers of the *2 haplotype, which includes the above mentioned var55146.

2.3.9 Influence of non-genetic factors in univariate and multivariate models

Next, univariate associations with clinical data of the Caucasian population were performed leading to some additional observations: patients with hepatocellular carcinoma (HCC) had a 1.3-fold ($P<0.05$) decreased PON1 protein expression, whereas in patients with cholangiocellular carcinoma (CCC) PON3 expression was 1.7-fold decreased ($P<0.05$). Interestingly, typical markers for liver-damage showed significant associations with paraoxonase phenotypes. C-reactive protein (CRP; a marker of inflammation) levels exceeding 8.2mg/l were associated with 2.3-fold lower PON1 ($P<0.01$), 2.6-fold lower PON3 ($P<0.01$) and 1.7-fold lower atorvastatin-lactone hydrolysis ($P<0.05$). Similarly, elevated levels of γ-glutamyl-transferase (GGT) (>64 in men / >36 in women) were linked to 1.4-fold decreased PON1 and PON3 expression, as well as, atorvastatin-lactone hydrolysis ($P<0.01$). Bilirubin levels higher than 1.2mg/dl were associated with 1.8-fold decreased PON3 expression ($P<0.01$) and 1.3-fold decreased atorvastatin-lactone hydrolysis ($P<0.05$). Significant differences between non-cholestasic and cholestatic patients (Nies et al., 2009) were identified for PON3 expression (decreased 1.6-fold, $P<0.01$) and atorvastatin-lactone hydrolysis (decreased 1.4-fold, $P<0.05$).

Presurgical treatment with omeprazole or pantoprazole was associated with a significantly lower PON3 mRNA and protein expression (1.3-fold, $P<0.05$; 1.6-fold, $P<0.01$) and a significantly lower activity (1.7-fold, $P<0.01$). Presurgical treatment with statins (N=7; atorvastatin or simvastatin) was associated with higher PON1 protein levels in treated *versus* untreated *PON1-108CC* carriers ($P=0.051$), but not in T allele carriers.

Finally, an approach to estimate the combined effect of all relevant genetic and non-genetic factors (see above) on atorvastatin-lactone hydrolysis and PON1 and PON3 expression was made. Therefore, multivariate linear models and step-wise model selection were applied on

the available genetic and non-genetic data. For this analysis, each polymorphism was tested with the genetic model (dominant, recessive or log-additive) with the most significant effect on the corresponding phenotype in univariate analyses. Figure 27 shows the fraction of observed population variability explained by polymorphisms, non- genetic factors or both.

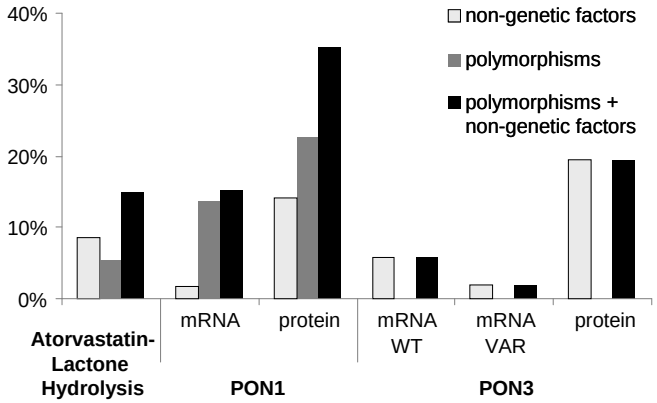


Figure 27: Contribution of genetic and non-genetic factors to PON1 and PON3 expression and activity. Percentage of total atorvastatin-lactone hydrolysis, PON1 and PON3 expression variation explained by multivariate linear models containing only non-genetic factors (white), only genetic factors (grey) or both (black). The bars indicate the coefficient of determination adjusted for the number of factors in the different models. Linear models were derived by step-wise model selection procedure using Akaike's information criterion.

Coefficients of determination adjusted for the number of factors in the model were then used to specify the fraction of phenotype variability explained by each polymorphism (in log-additive genetic model) and chosen non-genetic factors together. Atorvastatin-lactone hydrolysis variability could be explained to 15.0% by F21 (rs1322614), *PON3* -1091A>G promoter polymorphism (possibly via linkage with a not included *PON1* polymorphism) and the non-genetic factors sex, C-reactive protein, γ-glutamyl-transferase and cancer classification ($P<0.05$). *PON1 -108T>C* promoter polymorphism- together with non-genetic factors- determined 13.1% ($P<0.0001$) of PON1 mRNA (non-genetic factors: smoking status, cholestasis) and 35.3% ($P<0.0001$) of protein expression (sex, smoking status, C-reactive protein, γ-glutamyl-transferase). PON3 expression appears to be affected mainly by non-genetic factors only, explaining about 20% ($P<0.0001$) of protein variance, when smoking status, cancer classification, C-reactive protein, bilirubin level and γ-glutamyl-transferase are considered.

2.4 Healthy volunteer study for the influence of UGT1A3 haplotypes on atorvastatin-lactonization

As outlined in the previous chapters, this thesis identified genetic variations in *UGT1A3* and the *PON*-locus influencing formation or hydrolysis of atorvastatin-lactone *in vitro*, respectively. Only studies in patients are able to validate the importance of identified variations, first for *in vivo* lactone-levels, and second for atorvastatin response or risk for adverse events in treatment with atorvastatin.

Hence, to validate the role of *UGT1A3*-haplotypes on atorvastatin-lactonization, 56 volunteers in previously published pharmacogenetic studies (Keskitalo et al., 2008, Keskitalo et al., 2009) were genotyped (for VAF please refer to table 3, section 2.2.4) and associated to the corresponding haplotypes. *UGT1A3*-haplotype stratification of plasma concentration-time profiles and pharmacokinetic parameters of atorvastatin and metabolites exposed significant associations (figure 28, table 11). No significant differences were observed on the parameters C_{max} or t_{max} and only the half-life $t_{1/2}$ of o-OH-atorvastatin-lactone in *UGT1A3*2/**2 was significantly increased compared to *UGT1A3*1/*1*. Similarly, only the o-OH-atorvastatin-lactone $AUC_{0-\infty}$ was significantly increased (50%; $P < 0.05$) compared to *UGT1A3*1/*1*, whereas the $AUC_{0-\infty}$ values of atorvastatin or atorvastatin-lactone were not significantly affected by the haplotype. But when comparing the $AUC_{0-\infty}$ atorvastatin lactone to acid ratio, *UGT1A3*2* homozygotes (n=11) had 42% ($P < 0.01$) and **2* heterozygotes 37 % ($P < 0.05$) higher ratios than *UGT1A3*1/*1*. Similarly the $AUC_{0-\infty}$ of o-OH-atorvastatin-lactone/acid ratio was 33% higher in *UGT1A3*2/*2* carriers than in *UGT1A3*1/*1* individuals.
Though differences were also seen in *UGT1A3*3* or *UGT1A3*6* allele carriers, the numbers were too small to draw valid statistical conclusions.

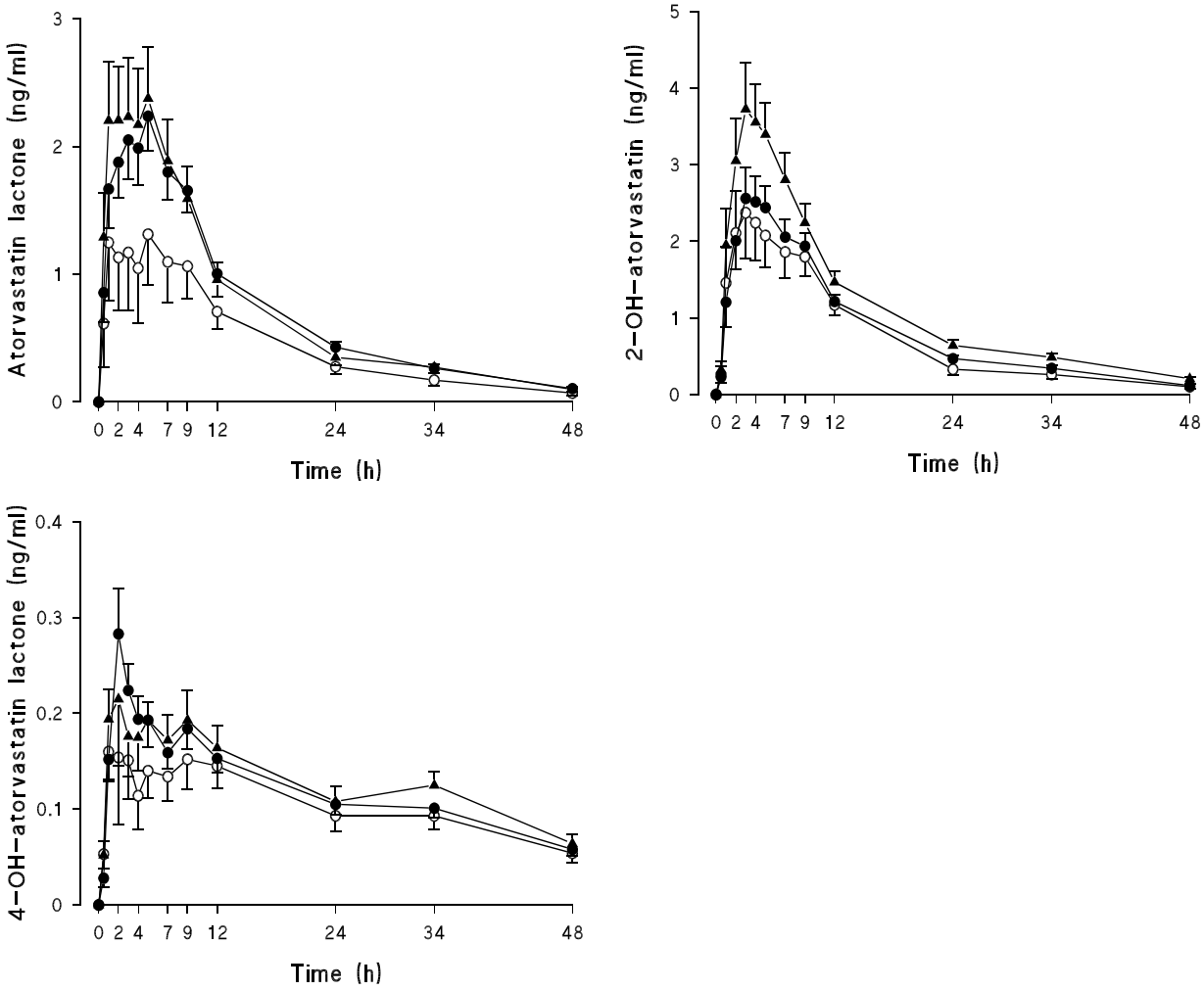


Figure 28: *UGT1A3* genotype and its Influence on atorvastatin and OH-atorvastatin lactone plasma concentrations *in vivo*. Estimated marginal mean values +/- SEM plasma concentrations of atorvastatin lactone, p-OH-atorvastatin lactone, and o-OH-atorvastatin lactone in individuals with different *UGT1A3* genotypes, following a single 20-mg oral dose of atorvastatin. Open circles, *UGT1A3*1/*1* genotype (n = 14); filled circles, *UGT1A3*1/*2* genotype (n = 25); triangles, *UGT1A3*2/*2* genotype (n = 11). Data from individuals carrying the *UGT1A3*3* or **6* allele were omitted for clarity.

Table 11: Pharmacokinetic variables of a single 20 mg oral dose of atorvastatin in 56 healthy white subjects in relation to the *UGT1A3* genotype.

***UGT1A3* genotype**	**C_{max} (ng/ml)**	**t_{max} (h)**	**$t_{½}$ (h)**	**$AUC_{0-\infty}$ (ng·h/ml)**	**$AUC_{0-\infty}$ ratio (lactone/acid)**
Atorvastatin					
**1/*1* (*n*=14)	6.2 (4.4–8.7)	0.75 (0.5–2.0)	10.6 (9.2–12.2)	26.3 (21.1–32.6)	
**1/*2* (*n*=25)	4.4 (3.5–5.6)	1.0 (0.5–5.0)	10.2 (9.3–11.2)	27.6 (23.8–32.0)	
**2/*2* (*n*=11)	6.5 (4.6–9.1)	0.5 (0.5–1.0)	10.1 (8.8–11.6)	27.6 (22.2–34.3)	
**1/*3* (*n*=3)	6.8 (3.1–15.0)	1.0 (1.0–2.0)	11.0 (7.9–15.1)	24.9 (15.1–41.0)	
**2/*3* (*n*=1)	7.1 (2.3–22.4)	1.0	10.8 (6.7–17.3)	34.6 (16.7–71.8)	
**1/*6* (*n*=2)	3.4 (1.3–9.2)	1.25 (0.5–2.0)	5.9 (3.9–8.8)[*,†,‡,§]	23.5 (12.5–44.3)	
Atorvastatin lactone					
**1/*1* (*n*=14)	1.5 (1.1–2.1)	4.5 (1.0–5.0)	11.5 (9.9–13.2)	21.9 (16.3–29.5)	0.83 (0.70–1.00)
**1/*2* (*n*=25)	2.1 (1.7–2.6)	5.0 (0.5–9.0)	11.1 (10.1–12.2)	31.6 (25.9–38.7)	1.14 (1.01–1.29)[**]
**2/*2* (*n*=11)	2.3 (1.7–3.2)	3.0 (0.5–5.0)	11.1 (9.6–12.8)	32.6 (24.2–44.0)	1.18 (0.99–1.42)[*]
**1/*3* (*n*=3)	2.0 (0.9–4.1)	3.0 (2.0–9.0)	11.5 (8.3–16.0)	28.7 (14.5–56.8)	1.15 (0.76–1.74)
**2/*3* (*n*=1)	2.3 (0.8–6.9)	5.0	6.7 (4.1–10.8)[*,†,‡]	30.5 (11.2–82.9)	0.88 (0.48–1.62)
**1/*6* (*n*=2)	1.8 (0.7–4.5)	3.0 (1.0–5.0)	8.2 (5.5–12.5)	18.1 (7.6–42.9)	0.77 (0.45–1.30)
o-OH-atorvastatin					
**1/*1* (*n*=14)	3.9 (2.8–5.5)	1.0 (0.5–5.0)	12.4 (10.7–14.4)	29.8 (24.3–36.7)	
**1/*2* (*n*=25)	2.3 (1.9–2.9)[*]	2.0 (0.5–5.0)	12.7 (11.4–14.0)	27.4 (23.8–31.4)	
**2/*2* (*n*=11)	3.0 (2.2–4.2)	1.0 (0.5–5.0)	13.9 (11.9–16.2)	33.5 (27.3–41.3)	
**1/*3* (*n*=3)	1.9 (0.9–4.0)	1.0 (1.0–1.0)	14.9 (10.5–21.1)	20.7 (12.9–33.2)	
**2/*3* (*n*=1)	2.6 (0.9–7.9)	1.0	9.2 (5.5–15.3)	35.4 (17.7–70.9)	
**1/*6* (*n*=2)	2.4 (0.9–6.3)	2.0 (1.0–3.0)	7.9 (5.1–12.3)[*,†,§]	36.6 (20.1–66.8)	
o-OH-atorvastatin lactone					
**1/*1* (*n*=14)	2.4 (1.8–3.2)	3.0 (3.0-5.0)	10.5 (9.1–12.2)	34.1 (27.0–43.2)	1.14 (0.98–1.33)
**1/*2* (*n*=25)	2.4 (2.0–3.0)	5.0 (3.0–9.0)	11.7 (10.6–12.9)	37.9 (32.3–44.4)	1.38 (1.25–1.54)
**2/*2* (*n*=11)	3.4 (2.5–4.5)	4.0 (2.0–7.0)	14.1 (12.2–16.2)[*,†]	51.1 (40.3–64.8)[*,†]	1.52 (1.31-1.78)[*]
**1/*3* (*n*=3)	2.0 (1.0–3.7)	3.0 (2.0–5.0)	12.7 (9.2–17.7)	27.6 (16.1–47.5)[‡]	1.34 (0.94–1.90)
**2/*3* (*n*=1)	2.0 (0.8–5.2)	5.0	7.4 (4.6–11.9)[‡]	31.7 (14.3–70.2)	0.90 (0.54–1.50)
**1/*6* (*n*=2)	1.7 (0.8–3.9)	4.0 (3.0–5.0)	7.6 (5.0–11.5)[†,‡‡]	24.8 (12.5–49.2)[‡]	0.68 (0.43–1.05)[*,††,‡‡,§]
p-OH-atorvastatin lactone					
**1/*1* (*n*=14)	0.20 (0.14–0.29)	2.0 (1.0-12.0)	29.8 (23.4–37.8)	7.0 (5.1–9.6)	
**1/*2* (*n*=25)	0.28 (0.22–0.36)	3.0 (1.0–9.0)	27.1 (23.0–31.9)	7.4 (5.9–9.2)	
**2/*2* (*n*=11)	0.25 (0.17–0.36)	2.0 (1.0–12.0)	27.9 (21.9–35.6)	8.1 (5.8–11.2)	
**1/*3* (*n*=3)	0.25 (0.11–0.58)	4.0 (2.0–5.0)	14.5 (8.4–25.2)[*,†,‡]	3.2 (1.5–6.7)[†,‡]	
**2/*3* (*n*=1)	0.29 (0.09–0.99)	34.0	36.4 (16.2–81.7)	9.7 (3.3–28.7)	
**1/*6* (*n*=2)	0.20 (0.07–0.58)	2.0 (1.0–3.0)	18.6 (9.2–37.3)	3.5 (1.4–8.9)	

2.5 Retrospective pharmacogenetic study on candidate atorvastatin metabolizing and transporting proteins

Access to samples and data of a clinically well documented study made it possible to analyze effects of identified genetic variants within a big study cohort on treatment response on lipid levels and survival rate of treated patients.

For retrospective genetic analysis in one assay, polymorphisms were chosen, which possibly affect atorvastatin-lactone levels as described in 2.2 and 2.3 and atorvastatin-hydroxylation by affecting CYP3A enzymes as shown in 2.1. Two polymorphisms of *UGT1A3* were selected allowing the identification of *UGT1A3*2* carriers. Homozygous *UGT1A3*2* carriers had shown a 2.1-fold increased lactone formation *in vitro* and a 42 % higher $AUC_{0-\infty}$ lactone to acid ratio in healthy volunteers. *PON3 -1091* (rs11767787) was included as A homozygotes had a 1.3-fold lower atorvastatin-lactone hydrolysis and a 1.8-fold lower PON1 expression compared to GG genotype. Additionally *PON3 -1091*A>G (rs11767787 and *PON3* F21 (rs13226149) was, together with non-genetic factors, found responsible for 15% ($P<0.05$) of the variability of atorvastatin-lactone hydrolysis. The only polymorphism significantly changing PON3 expression and atorvastatin-lactone hydrolysis was also included (var55146). *ABCG2c.421C>A* (rs2231142), which was reported to increase the mean plasma $AUC_{0-\infty}$ of atorvastatin in a healthy volunteer study (Keskitalo et al., 2009), was also analyzed. *CYP3A4*1B* (-392A>G; rs2740574), for which one study showed a significantly lowered metabolic activity (Rodríguez-Antona et al., 2005) and *CYP3A5*3* (rs776746), which explains the absence of CYP3A5 expression in many individuals (Kuehl et al., 2001) and may influence the individual atorvastatin hydroxylation were also included.

The study was performed with 1180 subjects from the "4-D study" (Wanner et al., 2005), a randomized multicenter study on type 2 diabetes mellitus patients having received 20 mg atorvastatin per day or matching placebo. The primary end point in this study had been defined as death from cardiac causes, nonfatal myocardial infarction and stroke. Clinical data included triglycerides, LDL and HDL levels for baseline and all visits, as well as, age, sex, bmi, smoking status, history of diabetes and cardiovascular disease and intervention, medication, count of several blood and liver parameters and systolic and diastolic blood pressure. Genotyping for the selected polymorphisms in 1180 samples by a 7-plex MALDI-TOF MS assay showed variant allelic frequencies (VAF) of the variant allele comparable to

those reported in public databases on Caucasians (dbSNP/Seattle SNP) or those determined in 150 Caucasian human liver samples (table 12).

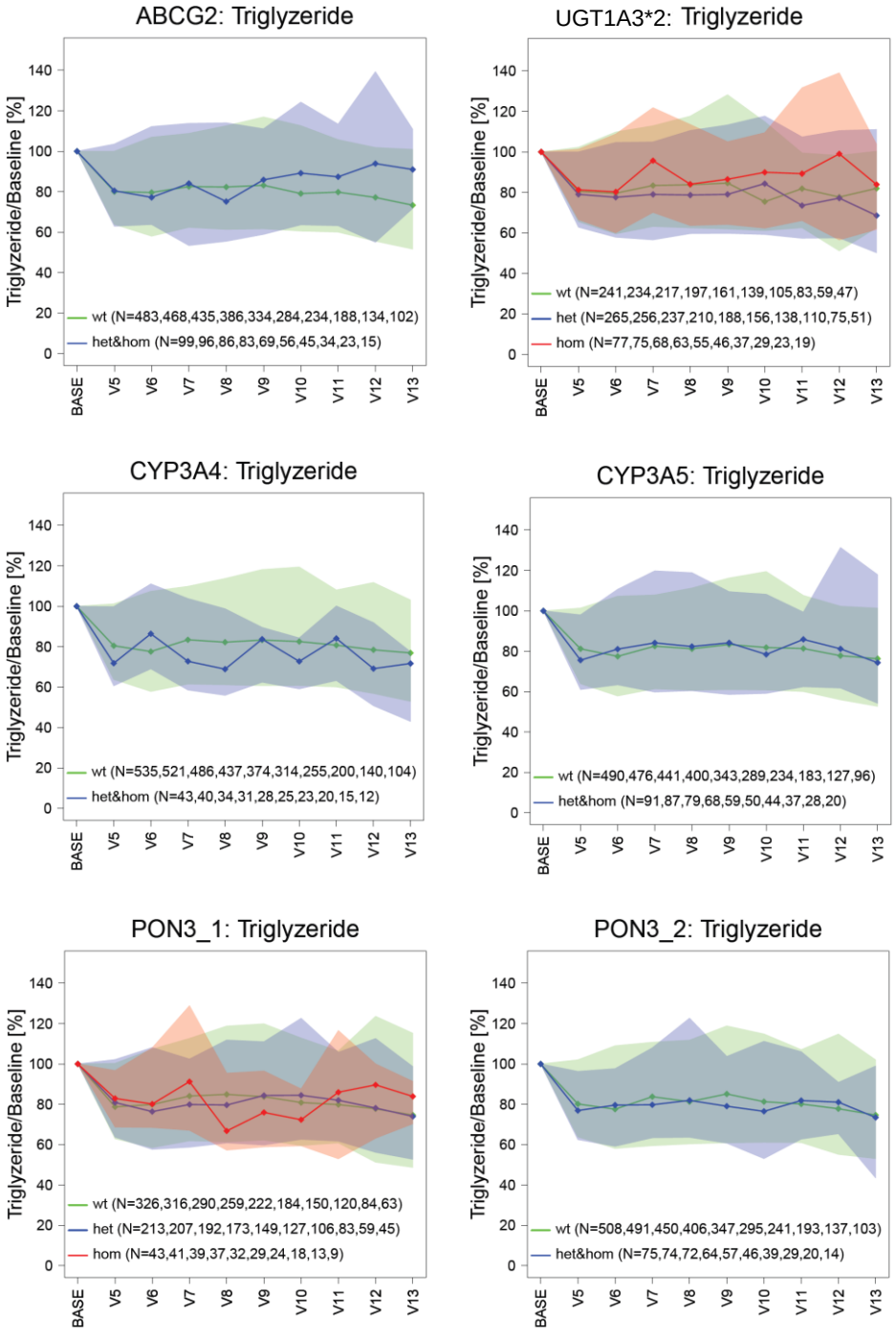
Table 12: Characteristics and variant allele frequencies (VAF) of SNPs determined in 1180 samples from the 4D-study.

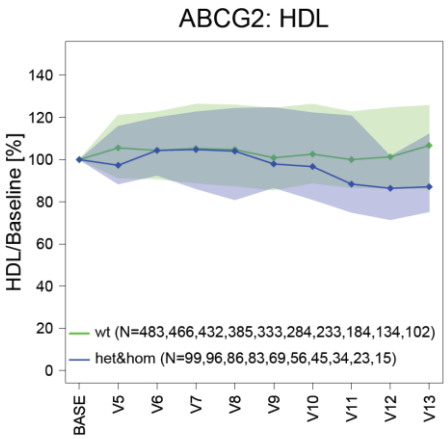
SNP	*Gene*	*SNP ID dbSNP or Seattle SNP*	*Genomic position*	*Base change*	*Residue change*	*Region*	*VAF*		
							4D-study	*IKP liver bank*	*dbSNP Build 132*
1	ABCG2	rs2231142	27688	C>A	Q141K	exonic	**0.101**		0.111
2	CYP3A4	rs2740574	4713	A>G	-	promoter	**0.038**	0.027	0.028
3	CYP3A5	rs776746	12083	G>A	-	intronic	**0.074**	0.037	0.036
4	PON3	rs11767787	3935	A>G	-	promoter	**0.259**	0.310	0.241
5	PON3	var55146	(53688)	G>A	-	intergenic	**0.076**	0.050	0.050 Seattle SNP
6	UGT1A3	rs1983023	144984	T>C	-	promoter	**0.406**	0.391	0.336
7	UGT1A3	rs45449995	146542	A>G	M270V	exon 1	**0.037**	0.040	0.014

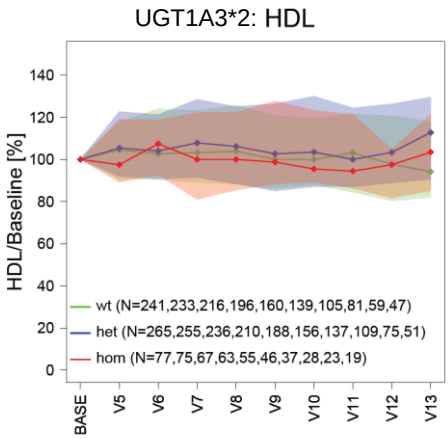
Six of the polymorphisms showed no deviation from Hardy-Weinberg equilibrium (HWE). PON3 var55146 was found to deviate significantly from HWE (p_{exact}=0.0001). Genotypes of this polymorphism were distributed as follows: *GG*:1010(1001); *GA*:146(165); *AA*:16(7) showing a high number of carriers of the variant allele as compared to the expected amount in brackets. An explanation for this bias of the sample selection is still to be found as problems with the gontyping assay could be excluded. No differences, which might possibly have biased the population like age, sex, bmi, systolic or diastolic blood pressure, diabetes duration, or baseline triglyceride or HDL levels were observed. Significant var55146 dependent differences were only observed for baseline LDL levels, which were decreased in heterozygous carriers (p=0.01).

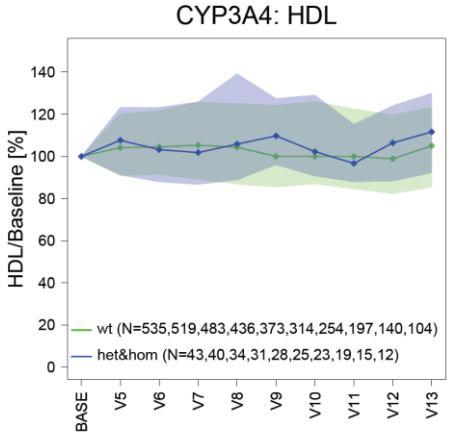
2.5.1 Influence of genotype on lipid levels (HDL, LDL, TGs) in atorvastatin receiving group

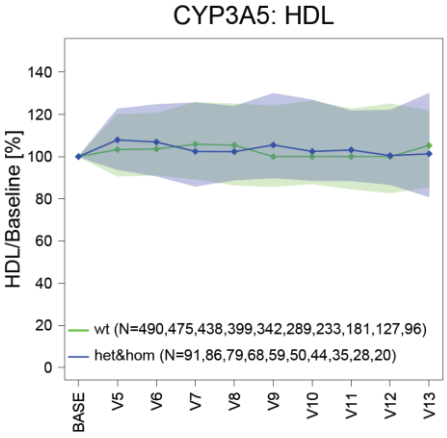
The influence of each genotype on the lipid levels of the atorvastatin receiving group was analyzed in comparison to the corresponding baseline level before the study, which was set as 100% (figure 29).

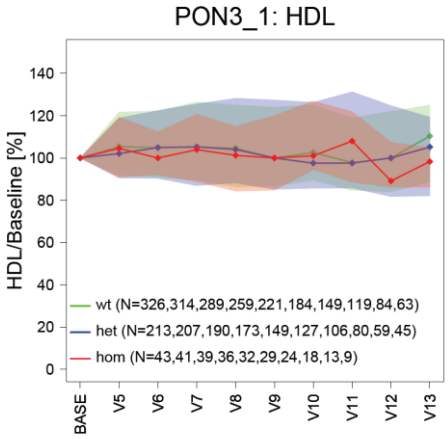


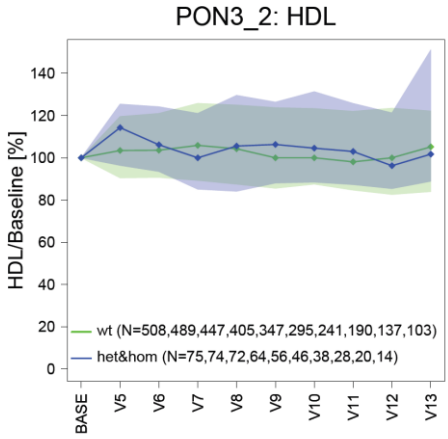

ABCG2: HDL
HDL/Baseline [%]
wt (N=483,466,432,385,333,284,233,184,134,102)
het&hom (N=99,96,86,83,69,56,45,34,23,15)
BASE V5 V6 V7 V8 V9 V10 V11 V12 V13

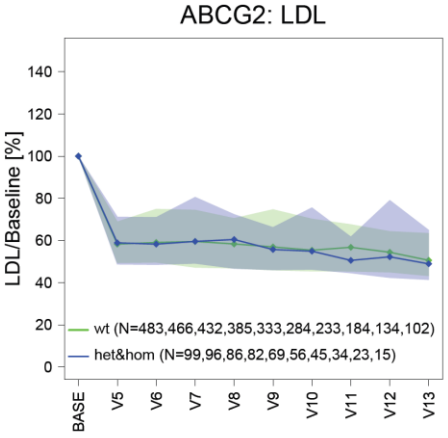

UGT1A3*2: HDL
HDL/Baseline [%]
wt (N=241,233,216,196,160,139,105,81,59,47)
het (N=265,255,236,210,188,156,137,109,75,51)
hom (N=77,75,67,63,55,46,37,28,23,19)
BASE V5 V6 V7 V8 V9 V10 V11 V12 V13

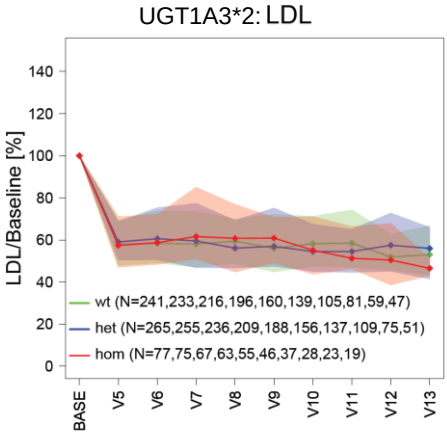

CYP3A4: HDL
HDL/Baseline [%]
wt (N=535,519,483,436,373,314,254,197,140,104)
het&hom (N=43,40,34,31,28,25,23,19,15,12)
BASE V5 V6 V7 V8 V9 V10 V11 V12 V13


CYP3A5: HDL
HDL/Baseline [%]
wt (N=490,475,438,399,342,289,233,181,127,96)
het&hom (N=91,86,79,68,59,50,44,35,28,20)
BASE V5 V6 V7 V8 V9 V10 V11 V12 V13


PON3_1: HDL
HDL/Baseline [%]
wt (N=326,314,289,259,221,184,149,119,84,63)
het (N=213,207,190,173,149,127,106,80,59,45)
hom (N=43,41,39,36,32,29,24,18,13,9)
BASE V5 V6 V7 V8 V9 V10 V11 V12 V13


PON3_2: HDL
HDL/Baseline [%]
wt (N=508,489,447,405,347,295,241,190,137,103)
het&hom (N=75,74,72,64,56,46,38,28,20,14)
BASE V5 V6 V7 V8 V9 V10 V11 V12 V13


ABCG2: LDL
LDL/Baseline [%]
wt (N=483,466,432,385,333,284,233,184,134,102)
het&hom (N=99,96,86,82,69,56,45,34,23,15)
BASE V5 V6 V7 V8 V9 V10 V11 V12 V13


UGT1A3*2: LDL
LDL/Baseline [%]
wt (N=241,233,216,196,160,139,105,81,59,47)
het (N=265,255,236,209,188,156,137,109,75,51)
hom (N=77,75,67,63,55,46,37,28,23,19)
BASE V5 V6 V7 V8 V9 V10 V11 V12 V13

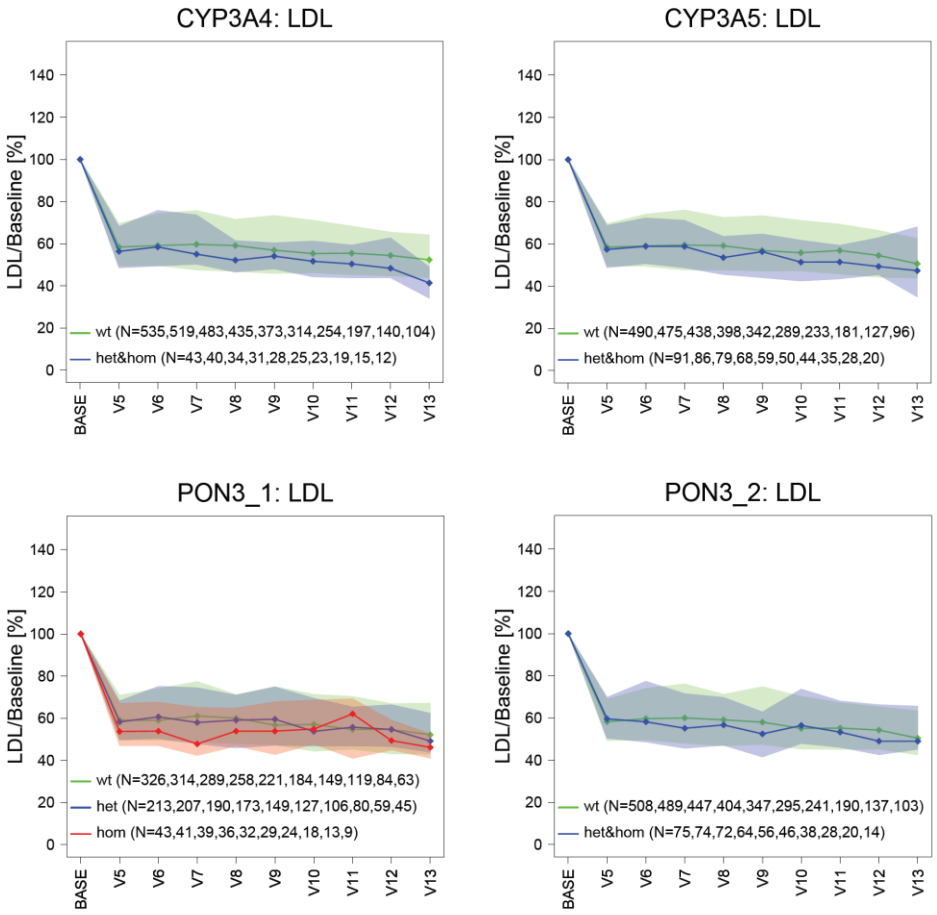
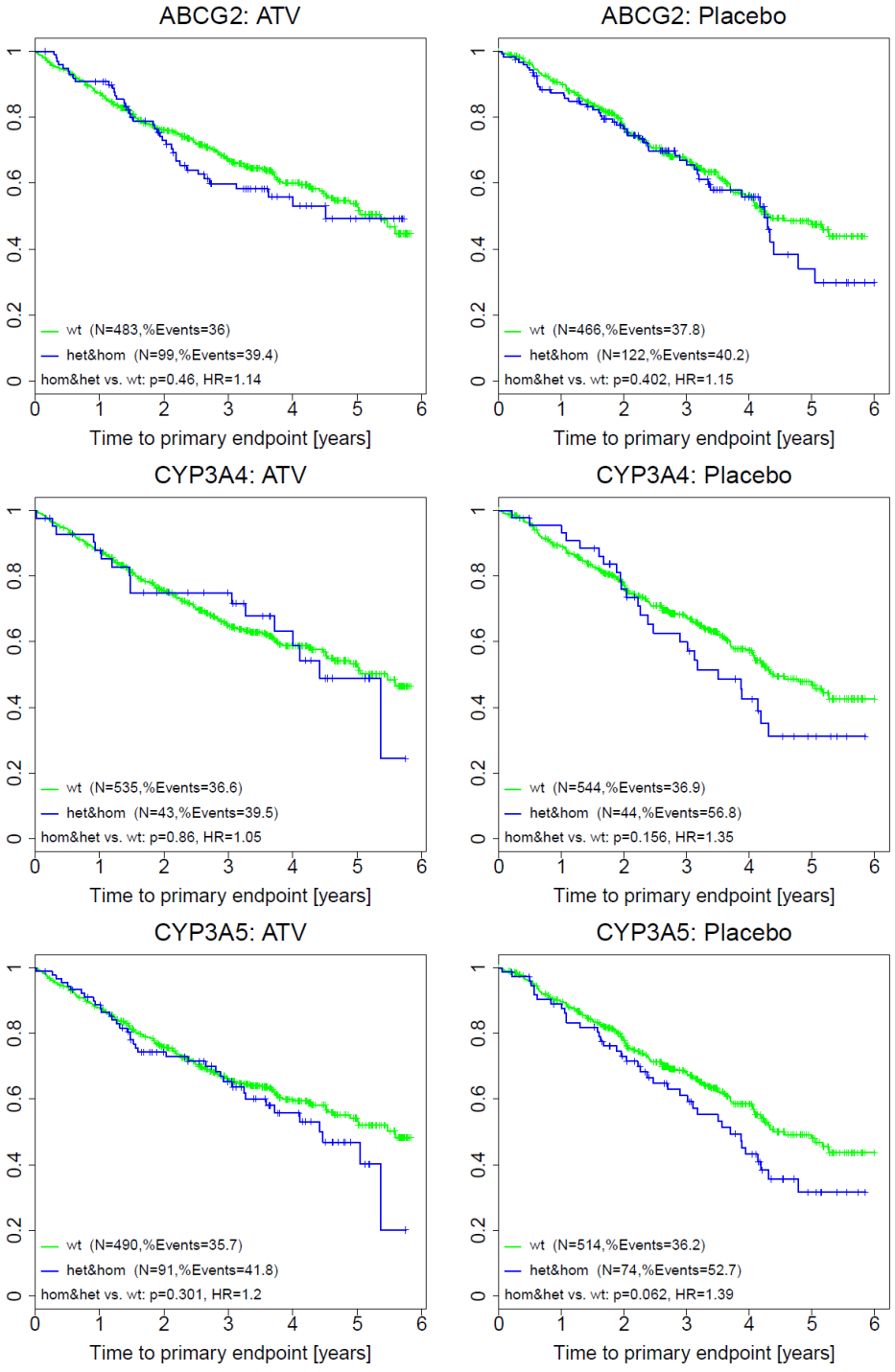


Figure 29: Triglyceride, HDL or LDL levels of participants reveiving atorvastatin compared to baseline level at study start associated for *ABCG2*, *UGT1A3*2*, *CYP3A4*, *CYP3A5* and *PON3* genotypes. Triglyceride/LDL /HDL profiles show median Triglyceride/baseline (LDL/baseline, HDL/baseline) levels (diamonds) at each time point for each genotype group (shaded areas are defined by 25%/75% quantiles). N lists the remaining population size at these dates for each genotype. Graphs were designed by Stefan Winter.

Statin treatment dependent effects like lowered triglyceride and LDL-levels were observed as expected. A significant difference according to genotype could be observed neither for triglyceride nor for HDL or for LDL levels.

2.5.2 Comparative Kaplan-Meier analysis of study endpoints in atorvastatin and placebo receiving groups

The primary endpoint of the study was defined as death from cardiac causes, fatal stroke, nonfatal cardiac infarction, non-fatal stroke, whichever occurred first. Survival within the 4D-study cohort was analyzed separately for 5 polymorphisms and for the *UGT1A3*2* haplotype (carriers of rs1983023 but not of rs45449995) respectively by Kaplan Meier analyses (figure 30).



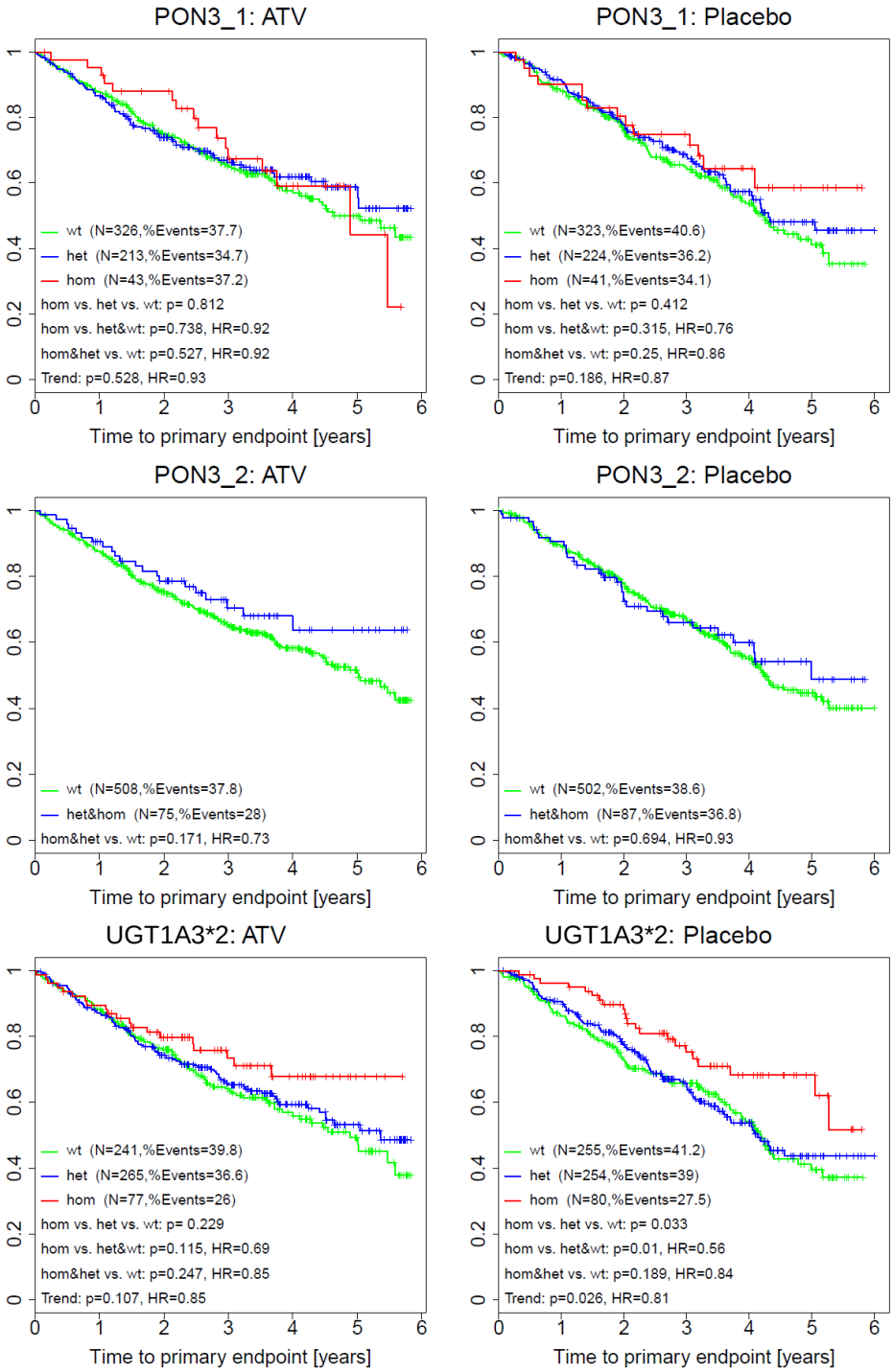


Figure 30: Kaplan-Meier plots for the primary study endpoint sorted for the analyzed genotypes. Where the amount of events made it possible, heterozygous and homozygous carriers were analyzed separately. Otherwise carriers were compared to non-carriers. Group size is given for each genotype (N), as well as, percentage of events per genotype (%Events). Significance was determined by log-rank tests and hazard ratios by cox-regressions. Graphs were designed by Stefan Winter.

Significantly increased survival was observed for homozygous carriers of *UGT1A3*2* of patients compared to *UGT1A3*1* and was most prominent in an analyses of homozygotes versus combined heterozygotes & wild-types (P<0.01). This was only observed in patients having received the placebo. The hazard ratio of homozygous carriers compared to the other

participants was determined as 0.56. A genotype dependent increased survival was also found in the atorvastatin receiving group, but was not significant. None of the other genetic variants tested in this study showed significant differences in Kaplan-Meier analyses.

2.5.3 *UGT1A3*2* associated increased survival in both groups

As this interesting association of *UGT1A3*2* homozygotes was not dependent on a treatment of atorvastatin, as it was only detected in the placebo group. Therefore, further analyses were performed on this genotype. Statistical analysis of the baseline study data of all participants revealed significant associations of wild-types/heterozygote carriers against homozygous *UGT1A3*2* carriers: most interestingly, this group had significantly less PTCA surgeries (percutaneous transluminal coronary angioplasty; P=0.0079; OR=0.35) and also less CAD (coronary artery diseases; P=0.0243; OR=0.66; both one sided fishers exact tests with no correction for multiple testing) before the start of the study. An association of the genotype with myocardial infarction was also tested but no difference was detected. An observation, which might provide some evidence for the way how is shown in figure 31.

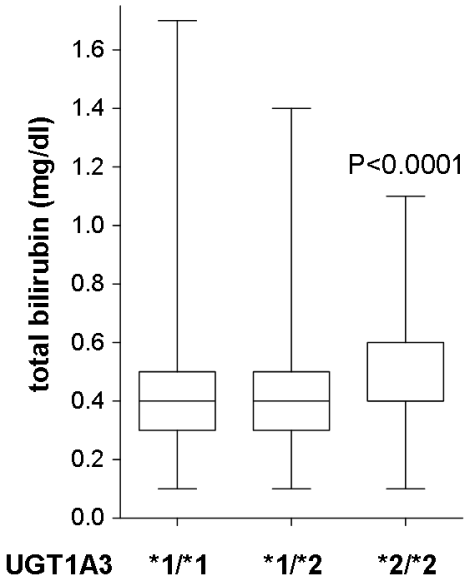


Figure 31: Level of total bilirubin (mg/dl) calculated for *UGT1A3*2* genotype. Plots indicate the median (horizontal line) and statistical significance according to Wilcoxon-Mann-Whitney as compared with wild-type is indicated.

Bilirubin, a known antioxidant (Stocker et al., 1987), was significantly increased in *UGT1A3**2 homozygotes, only. In a Kaplan-Meier analysis of the primary study endpoint of all study participants, significantly increased survival was detected in the same group, the *UGT1A3*2* homozygotes (figure 32).

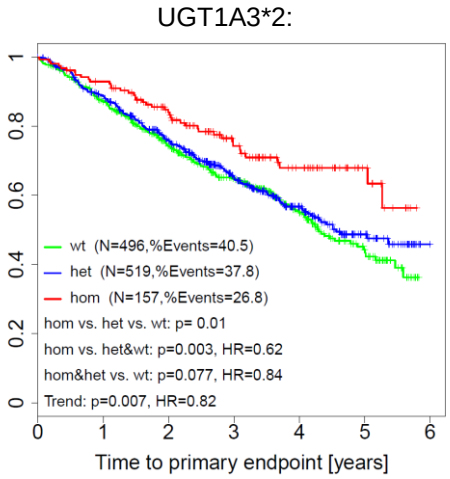


Figure 32: Kaplan-Meier plot of the primary study endpoint of all study participants for *UGT1A3*2*. Group size is given for each genotype (N), as well as, percentage of events per genotype (%Events). Significance was determined by log-rank tests and hazard ratios by cox-regressions. Graph was designed by Stefan Winter.

3. Discussion

Atorvastatin belongs to the class of HMG CoA reductase inhibitors and is a frequently used and relatively safe drug. The occurance of adverse events is rare, but hepatotoxicity, myopathy and rhabdomyolysis are well known. Inter-individual variation in statin response may in part be explained by genetic variations. Many genes were investigated as determinants for statin response and influential loci were identified affecting cholesterol absorption (*ApoE, ABCG5, ABCG8*), cholesterol synthesis (*HMGCR*) and lipoprotein catabolism (*ApoB, LDLR*). In addition, loci within statin metabolizing cytochrome P450 genes were found, which affected statin responsiveness (Kajinami et al., 2005). However, as many studies focused only on single genes, reported results varied considerably.

An involvement of atorvastatin- lactone metabolites in the development of adverse events recently became plausible, as significantly increased atorvastatin lactone was observed in myopathy patients (Hermann et al., 2006). Additionally, it was shown by *in vitro* studies on human skeletal muscle cells (Skottheim et al., 2008) and on primary rainbow trout hepatocytes (Ellesat et al., 2010) that atorvastatin lactone was more toxic than the acid form. Therefore, this study focused on the identification of genetic variations affecting the levels of atorvastatin-lactone as possible risk factors for statin therapy failure or statin-related adverse event and indeed identified influential loci in *UGT1A3, PON1* and *PON3*.

Additionally, the effects of polymorphisms within multiple candidate loci related to atorvastatin metabolism and transport (*CYP3A4, CYP3A5, UGT1A3, PON1, PON3* and *ABCG2*) were tested in a great number of study participants. This retrospective pharmacogenetic analysis used samples from patients having received atorvastatin in a study on type 2 diabetes mellitus patients on dialysis ("Die deutsche Diabetes Dialyse Studie"-4D study).

3.1 Enzymes affecting hydroxylation, lactonization and lactone-hydrolysis and their population characteristics

First, this thesis targeted the identification of genes involved in the metabolism of atorvastatin and, therefore, possibly related to changes in certain metabolite levels. Hydroxylation via CYP3A enzymes was known as a major pathway in the metabolism of atorvastatin (Jacobsen et al., 2000). Using 11 recombinantly expressed CYP enzymes it was shown that hydroxylation is predominantly catalyzed by CYP3A4 and CYP3A5. Less than 2% of the

CYP3A4 activity in p-OH metabolite formation was determined for CYP2C8 as reported previously (Jacobsen et al., 2000), whereas no o-OH metabolite was formed by this enzyme. The enhancing effect of cytochrome b5 on CYP3A4 and CYP3A5 activity for certain substrates described previously (Yamaori et al., 2003) was also detected in the hydroxylation of atorvastatin. p-OH- and o-OH-atorvastatin formation was ~6-fold higher in recombinant CYP3A4 with co-expressed cytochrome b5. o-OH-atorvastatin formation by recombinant CYP3A5 with co-expressed cytochrome b5 was similarly increased 6- to 8-fold, but surprisingly p-OH-metabolite formation was only minimally higher compared to the activity of recombinant CYP3A5 without expression of this additional electron donator.

As mentioned in 1.4, polymorphisms of *CYP3A4* and *CYP3A5* have been reported to significantly affect the activity or expression of these enzymes. But recent work in our group showed that none of the analyzed 11 CYP3A4 and 5 CYP3A5 polymorphisms including *CYP3A4*1B* (rs2740574), *CYP3A5*3* (rs776746) or the recently suggested biomarker for *CYP3A4* expression and statin response, rs35599367 (Wang et al., 2010) significantly changed the formation of p-OH- or o-OH-atorvastatin from the parent drug in 150 human liver samples. As a consequent step, genetic variations of other enzymes involved in atorvastatin metabolism were analyzed for potential influence on atorvastatin pharmacokinetics.

The conversion of atorvastatin to atorvastatin-lactone, a pharmacologically inactive compound, may occur chemically at low pH or enzymatically. Enzymatic conversion was described to be catalyzed mainly by UDP-glucuronosyltransferases UGT1A1 and UGT1A3 (Goosen et al., 2007). In using five different statin-acids, an important role of UGT mediated conversion was shown for atorvastatin followed by rosuvastatin. Experiments with 9 recombinant UGT isozymes supported that atorvastatin-lactonization is catalyzed by UGT1A1 and UGT1A3. Noticeable is that the observed activity of UGT1A3 was 3.7- to 9.3-fold higher than UGT1A1 at 1 and 10µM atorvastatin, respectively. Whereas for UGT1A3 a K_m of 12µM and V_{max} of 212pmol/min/mg was detected an in line with previous data (K_m = 12µM, V_{max} = 74pmol/min/mg, Goosen et al., 2007), V_{max} for UGT1A1 could not be reached. Though this clearly proves UGT1A3 to be a highly specific enzyme in atorvastatin-lactone formation, the *in vivo* importance of this finding also depends on the relative expression levels of enzymes involved in this transformation step. A study comparing the mRNA levels of 9 UGT1A and 7 UGT2B levels in 25 human livers showed a relative quantity of 11.3 % of

UGT1A1 and only 1.4 % of UGT1A3 (Izukawa et al., 2009). Therefore UGT dependent lactone formation in human liver microsomes, as well as, the mRNA and protein expression levels of UGT1A1 and UGT1A3 were determined in a Caucasian population (N = 150) and used for correlation analyses. Results clearly showed significant correlation with UGT1A3 mRNA and protein expression, but not with UGT1A1 expression. In conclusion, UGT1A3 was identified as the enzyme catalyzing atorvastatin-lactone formation based on literature data, comparative activity in 9 different isozymes, a low K_m combined with a high V_{max} and a significant correlation of atorvastatin-lactone formation and UGT1A3 expression in a Caucasian population.

The enzymatic conversion of atorvastatin-lactone to the acid conformation has not been analyzed before to the best of our knowledge. In order to identify the enzymes responsible for this conversion, expression profiles of 17 candidate esterase genes were screened for correlation with atorvastatin-lactone hydrolysis activity, revealing several positively and negatively correlated genes. Although PON1 and PON3 showed the strongest correlations, butyrylcholinesterase (BCHE) and carboxylesterase 1 (CES3) were also, but less strongly correlated positively. This study did not follow up on these other correlated candidate esterase genes, but it may be assumed that they contribute to minor extent to the overall atorvastatin-lactone hydrolysis in liver.

This primary approach was followed by real-time PCR mRNA quantification of PON1 and PON3 based on detailed transcript analyses leading to some additional observations on the mRNA level. Database information had suggested the existence of up to 4 and 9 transcript variants for PON1 and PON3, respectively. In contrast to this, an RT-PCR based analysis identified in addition to the wild-type transcripts of both paraoxonases only a single, so far unknown splice variant of PON3 with an open reading frame of 144bp. This variant was estimated to be about 3-fold less expressed and, as it was not detected on western blots, most probably either not translated or post-translationally degraded. Studies on the protein level extended previous knowledge showing PON1 expression in liver (Gonzalvo et al., 1998), by the observation of principally microsomal localization of human PON3.The fact that PON1 and PON3 correlated similarly (r_s = 0.60 and 0.62, respectively) to atorvastatin lactonase activity was in agreement with Western blots showing comparable signal intensities for PON1 and PON3 in pooled liver microsomes, although absolute quantification could not be performed due to the lack of standards. When protein levels of both paraoxonases were

combined, they accounted for ~46% of the ~122-fold variability of atorvastatin lactone hydrolysis in the liver samples. Moreover, the absolute quantification of transcript levels revealed PON3 normal transcript to be 5.1-fold higher expressed than PON1. This finding contrasts to opposite proportions reported for circulating, HDL-bound PON1 and PON3 in the bloodstream of 96 mg/l and 1.8 mg/l, respectively (Aragones et al., 2011). This seems surprising, but might be explained by a strongly favored secretion of PON1 due to its more pronounced glycosylation. This is supported by the fact that N-glycans constitute sorting signals for proteins (André et al., 2009).

3.2 Polymorphisms affecting lactonization and lactone-hydrolysis

This study identified UGT1A3, PON1 and PON3 as the enzymes significantly affecting the levels of atorvastatin-lactone by controlling the formation or the hydrolysis of this compound. Consequently, the next steps included an exploration of the genetic variability within the *UGT1A*- and the *PON*-locus and the determination of a possible influence on population variability of enzyme expression levels and atorvastatin-lactone formation or hydrolysis.

In the *UGT1A*-locus, the increased formation of atorvastatin-lactone was indeed associated with one or two *UGT1A3*2* alleles and the result of increased UGT1A3 expression. Supporting the role of UGT1A3, lactonization was inversely correlated with the *UGT1A1*28* genotype, which further excluded UGT1A1 as a candidate. This could be explained by high linkage within the *UGT1A*-locus. The way, in which lactonization is affected by the *UGT1A3*2* polymorphisms, is surprising. Previous studies had exclusively studied effects in recombinant expression systems. A study in a Japanese population had discovered four non-synonymous and two silent polymorphisms. Among five alleles with different combinations of amino acid variants functionally expressed in COS-7 cells, a variant corresponding to *UGT1A3*2* (*W11R + V47A*) showed in comparison to the wild-type allele a 3.7-fold increased intrinsic clearance ($Cl_{int} = V_{max}/K_m$) (Iwai et al., 2004). Estrone was used as a substrate in these assays and quantified via thin layer chromatography. Whereas neither differences in the amount of expressed protein nor increased maximal velocity were detected, the higher activity was due mainly to a decreased Michaelis-Menten constant (K_m). Another study identified 17 SNPs including 7 in the promoter region and 6 amino acid changes in 249 Caucasians (Caillier et al., 2007). This group, using also estrone as a substrate, analyzed protein variants in stably transfected HEK-293 cells, which unfortunately, due to variable chromosomal gene insertions, makes reliable conclusions on expression differences difficult. In contrast to the first study they found in *UGT1A3*2* variants a moderately decreased V_{max} but an unchanged

K_m. In summary, both studies did not indicate significant changes in expression or V_{max} associated with *UGT1A3*2*. In opposition to that, results of this study showed a substantial increase in mRNA (4.4-fold) and protein (7.3-fold) associated with homozygous *UGT1A3*2* genotype. Highly interesting considering the controversial results found with estrone are outcomes of this study with estrone and in addition to this also with the bile acids deoxycholic- (DCA) and lithocholic-acid (LCA). Applying the same recombinant expression system as the first study, a 7.7-fold increased protein expression of *UGT1A3*2* compared to *UGT1A3*1* were observed, which clearly reflects the observed increased protein expression of *UGT1A3*2* homozygous in the population. The specific activity of the recombinantly expressed variants with DCA and LCA was 5.5-fold and 5.8-fold reduced, respectively. Taking now into account the 7.7-fold increase in expression and the decrease in specific activity of *UGT1A3*2*, the results perfectly matches the mean 1.7-, 2.8- or 2.4-fold increased estrone-, DCA- or LCA-glucuronidation or 2.1-fold atorvastatin-lactonization observed with *in vitro* experiments with homozygous *2 carriers. In summary this showed that the decreased specific activity of this variant is overcompensated by an increased expression.

Genotype-phenotype analyses including further alleles show some interesting details on the possible mechanism of altered expression. The *UGT1A3*3* allele was associated only with a minimally increased mRNA- and an unchanged protein-level. The activity was non-significantly increased with the substrates atorvastatin, estrone and LCA, but 2-fold significantly increased in DCA-glucuronidation compared to *UGT1A3*1/*1*. The haplotype structure is very similar to the **2* allele except that a promoter polymorphism *-751 T>C* and the *V47A* amino acid change are missing. Focusing on promoter variants, luciferase reporter gene transfection of another group had revealed ~60 % lower promoter activity of **2* and **3* alleles in HepG2 cells making a role of the promoter variants unlikely (Caillier et al., 2007). In line with this, data from recombinant expression of the *2 variant carrying only the exonic polymorphism *W11R*, *E27* (rs6706232), *V47A* and *A159* (rs7574296) while showing a 7.7-fold increased expression pointed clearly to an increased mRNA or protein stability caused most probably by the *V47A* variation. Future work should clarify, whether increased mRNA stability is the reason for this observation.

The *UGT1A3*6* allele was associated with high mRNA and protein expression, but with no changes in any of the four tested substrates in comparison to the wild-type allele. This can be explained by the additional *M270V* amino acid variant, for which a previous study had shown

a complete loss of activity (Caillier et al., 2007). A novel allele *UGT1A3*12*, which resembles the **2* allele and carries an additional *M208L* amino acid variant, was detected once in combination with **2a* and had a similar individual levels in expression and activity phenotypes as *UGT1A3*2*. Finally the allele *UGT1A3*8a* was detected in two individuals, once in a combination with **1* and once with **2a*. The **8a/*1* carrier had low phenotypes in line with a previously shown loss of activity of **8a* whereas **8a/*2a* had increased phenotypes, which might be explained by the presence of the **2a* allele. A comparison of these alleles offers some insight into the functional consequences of the identified polymorphisms. However, only **2* is common within the population and was associated with increased atorvastatin-lactone formation and might therefore be important in atorvastatin-related adverse events.

Next this study focused on genetic variability of the *PON*-locus to explain the previously discovered high variability in atorvastatin-lactone hydrolysis. Following the previously stated hypothesis, genetic variants might explain observed changes in lactone levels. Although several SNPs at the *PON*-locus on chromosome 7 have been previously studied (Carlson et al., 2006, Wang et al., 2003) this was the first study to look at *PON1* and *PON3* together, which included a thorough genotyping of *PON3* through analysis of 35 SNPs covering the whole gene. An assessment of the linkage patterns of these SNPs made it possible to define 13 PON1-PON3 haplotypes. Based on functionally relevant SNPs, these haplotypes could be grouped into 7 major haplotypes. Of interest, these did not include the two amino acid variants L55M and Q192R. The latter variation, Q192R, affects the active histidine dyad of PON1, as crystal structure analysis had revealed (Harel et al., 2004). However the catalytic consequences are not consistent, as the kinetics of some substrates are accelerated (e.g. paraoxon) whereas others are slowed down (e.g. diazoxon) or not affected (e.g. phenylacetate; Costa and Furlong, 2002). According to the results on atorvastatin-lactone hydrolysis in liver microsomes, this substrate belongs to the last group and is not affected by either of these two variants.

PON1 promoter polymorphisms had, in contrast to L55M and Q192R, a clear effect on expression of PON1 but not PON3. *-108T>C* explained 23% of PON1 protein expression in liver, in very good agreement with a previous estimation that this SNP accounts for ~22-25% of variation in PON1 serum expression in white adults (Costa and Furlong, 2002) and appeared to be the most influential SNP for atorvastatin-lactone hydrolysis and PON1

expression. Evidence for *-108T>C* as a causal variant includes ~2-fold higher activity of the C allele in a reporter gene assay (Leviev and James, 2000), presumably due to disruption of a binding site for the transcription factor Sp1 in the T-allele (Deakin et al., 2003), or a binding site for the aryl hydrocarbon receptor, AhR (Gouédard et al., 2004). In addition, carriers of the high-expressor C-allele were found to show reduced LDL oxidation following treatment with atorvastatin or simvastatin presumably due to PON1 induction via promoter binding of SREBP-2 near to the Sp1 site (Sardo et al., 2005,Deakin, Leviev, Brulhart-Meynet, et al., 2003). This is supported by the observation that statin treatment in liver donors (N=7, atorvastatin and simvastatin) was indeed associated with a trend towards higher PON1 protein levels in treated *versus* untreated *PON1-108CC* carriers (P=0.051). *Vice versa*, protein levels of T allele carriers on statins were similar or lower than untreated patients with T allele. In summary these observations support *-108T>C* as a causal variant influencing PON1 expression and atorvastatin lactone hydrolysis, as well as, inducibility of the PON1 promoter. The PON1 promoter polymorphisms *-832G>A* and *-1741G>A* were similarly associated with increased PON1 protein and atorvastatin-lactone hydrolysis. It is highly possible that these associations are due to extensive linkage between the three promoter polymorphisms, although one study reported 1.7-fold higher activity of the *-832A* allele in a reporter gene assay (Leviev and James, 2000).

PON3 expression was not affected by the common variations analyzed in this study. However, one variant located about 12kb downstream of the last PON3 exon and about 23kb upstream of PON1 (var55146, VAF=5%) was associated with increased protein expression of only PON3, and with marginally increased atorvastatin-lactone hydrolysis, but not with altered mRNA. It remains to be clarified, whether this association is based on the high linkage to six intronic SNPs (figure 22) or (an) unidentified polymorphism(s) or on a downstream enhancer element of PON3. Interestingly, most of the identified *PON3* promoter and intronic polymorphisms, as well as, F21 (rs13226149) and A99 (rs1053275) were part of a block of high linkage not affecting PON3 expression. But polymorphisms of this linkage block were clearly associated with increased expression of PON1 and an increased hydrolysis of atorvastatin-lactone.

In contrast to the analyzed PON3 polymorphisms, multivariate analysis showed that non-genetic factors were clearly associated with changes in PON3 expression (figure 27). Observed associations with C-reactive protein, γ-glutamyl-transferase, bilirubin, cholestasis or

treatment with omeprazole or pantoprazole leading to decreased PON3 expression have not been described before. However, decreased expression of PON1 in patients with elevated C-reactive protein and γ-glutamyl-transferase and down-regulation of PON1 expression in response to inflammatory cytokines has been described before (Mackness et al., 2006, Araoud et al., 2010, Han et al., 2006, Van Lenten et al., 2001). PON1 activity has therefore been suggested as a marker for liver impairment (Marsillach et al., 2009). The associations with hepatocellular carcinoma (PON1) and cholangiocellular carcinoma (PON3) also seem to be plausible as paraoxonases have a protective effect against oxidative stress, which plays an important role in chronic liver diseases leading to liver cirrhosis and the development of carcinomas (Camps et al., 2009).

In summary, PON1 and PON3 were identified as the major enzymes for hydrolysis of atorvastatin-lactone in human liver. The analyzed data show that PON1 expression is controlled mainly by the linked promoter polymorphisms *-108T>C*, *-832G>A*, and *-1741G>A*, and the former, of which presumably represents the causal variant.

3.3 In vivo relevance of *UGT1A3* and *PON*-locus polymorphisms

As a final point, the *in vivo* relevance of *UGT1A3* and *PON*-locus polymorphisms for the treatment with atorvastatin was tested in this work. In the past, several studies have concentrated on the identification of candidate genes or polymorphisms possibly affecting the pharmacokinetics of statins and associated with statin response or statin-related adverse events. For example, *SLCO1B1* V147A (rs 4149056) CC had a 144% greater AUC of atorvastatin and a 100% greater AUC of o-OH-atorvastatin than subjects with the TC or TT genotype (Pasanen et al., 2007) and *ABCG2* Q141K (rs2231142) AA genotype had a 72% larger AUC of atorvastatin and a 94% larger AUC of atorvastatin-lactone than CC carriers (Keskitalo et al., 2009). These studies had been performed on groups of healthy volunteers having received a single dose of 20mg atorvastatin. Retrospectively genotyping samples from these studies, this work could show a 42% increased atorvastatin-lactone to acid AUC ratio, a 33% increased o-OH-atorvastatin lactone to acid AUC ratio, as well as, 50% increased o-OH-atorvastatin lactone in *UGT1A3*2* homozygotes compared to *UGT1A3*1* homozygotes. So, *UGT1A3*2* greatly affects not only plasma-lactone levels but also OH-lactone levels of atorvastatin, though the influence wasn't as distinct as the effects of the previously published transporter polymorphisms. Furthermore, genome-wide association studies focusing on the influences of polymorphisms on lipid-levels under treatment with statins identified SNPs in various genes including *HMGCR*, *SLCO1B1* or *APOE* (Barber et al., 2010, Link et al., 2008,

Thompson et al., 2009). Interestingly, the second study also identified a *PON1* polymorphism (rs854571; -832G>A) associated with changed response in LDL-cholesterol.

Therefore, this thesis analyzed the effects of identified candidate polymorphisms of *UGT1A3*, *PON1* and *PON3*, as well as, candidate polymorphism of genes also involved in the metabolism or transport of atorvastatin and its metabolites (*ABCG2*, *CYP3A4* and *CYP3A5*) on lipid levels or on the study endpoint. The study cohort consisted of 1180 samples from "Die deutsche Diabetes Dialyse Studie" (4D-study), from patients with type II diabetes on dialysis having received either atorvastatin or placebo and followed-up for a mean of 4 years. Significant, genotype-dependent differences in LDL-cholesterol, HDL-cholesterol or triglyceride levels could be observed neither in the placebo nor in the atorvastatin receiving group. Additionally, in Kaplan-Meier survival analyses, only *UGT1A3*2* homozygotes showed significantly less endpoints in the placebo group and when both groups were analyzed in combination. This observation clearly excludes an atorvastatin dependent effect.

A possible hint to explain this curious observation was, that *UGT1A3*2* homozygotes also had significantly increased serum-bilirubin levels. Bilirubin is known to be a highly potent antioxidant (Stocker et al., 1987). Oxidation of LDL is known to play an important part in the development of rupture prone atherosclerotic plaques. Modified LDL having infiltrated the intima of arteries leads to the activation of inflammation processes, which is an important factor in plaque formation. Additionally, plaque ruptures mainly occur where inflammation processes have weakened the fibrous plaque cap (Hansson, 2005). Therefore it is likely, that antioxidants can help to prevent LDL oxidation and by that atherosclerotic processes. Several studies observed linkage between serum bilirubin, bilirubin controlling genes (like UGT1A1) and cardiovascular disease. Several studies found a protective effect of a genotype (*UGT1A1*28*) leading to reduced expression of UGT1A1. The Framingham study found an odds ratio of ~0.36 for CVD in *UGT1A1*28* carriers (Johnson et al., 2009) and Lin et al., 2009 an odds ratio of ~0.24 in carriers of *UGT1A1*-locus polymorphisms linked to *UGT1A1*28*. On the other hand, some studies also found no cardiovascular protective effects of these *UGT1A1*-polymorphisms (Rotterdam, Bosma et al., 2003; ECTIM, Gajdos et al., 2006; CAVASIC, Rantner et al., 2008). In 2009, two genome-wide association studies for serum bilirubin levels (Johnson et al., 2009; rs6742078, Sanna et al., 2009; rs887829), one of them on the Rotterdam and Framingham study collectives, and the second one on 4300 Sardinian individuals, identified the *UGT1A1*-locus ($P < 5x10^{-324}$; $p = 6.2x10^{-62}$) as being

linked with increased serum bilirubin. With the top *UGT1A1* SNP it was possible to explain 18% of bilirubin variability. *UGT1A3* rs1983023 polymorphism, which was genotyped in the 4D study cohort was also found in one of the studies to influence bilirubin levels ($P = 2.8x10^{-307}$). Bilirubin is glucuronidated by UGT1A1, but it is no substrate of UGT1A3 (Green et al., 1998). Therefore it seems likely that *UGT1A3* rs1983023 (promoter -*751T>C*) is influencing bilirubin levels by high linkage with *UGT1A1*28* (figure 12) leading to the observed increased survival of carriers of *UGT1A3*2* (carrying rs1983023, but not rs45449995) with an odds ratio of ~0.62 in the 4D-study cohort.

Paraoxonases were also described as potent antioxidants with a protective effect in metabolic syndrome (Sentí et al., 2003). Additionally this study could show that PON1 expression was clearly affected by polymorphisms of the *PON*1* haplotype (see 2.3.8). The fact that none of the *PON*-locus polymorphisms influenced survival, either via the antioxidant capacity of paraoxonases or via their role in atorvastatin metabolism seems to rule out an important antioxidative role of these enzymes and variability within the *PON*-locus on the survival of atorvastatin treated patients. However, results from a recent study cast doubt on this conclusion. Rosenblat et al. (Mackness et al., 2008) could show that high glucose levels release PON1 from HDL particles and decrease its protein stability, as well as, its activity. In line with these results they observed significantly decreased PON activity in diabetes patients compared to control patients. Although no PON activity or expression had been determined in the 4D-study, one can speculate that participants of the 4D-study had low paraoxonase levels. Hence, possible *PON*-locus genotype dependent effects might not be detectable in a population of diabetes patients. In conclusion, *PON*-locus polymorphisms should be tested in a non-diabetes cohort to avoid the above described limitations of the 4D-study. In addition, the specific clinical factors of the 4D-study, like a high age and BMI, as well as, the high risk for cardiovascular diseases and the fact that the study only focused on diabetes patients (Wanner et al., 2005) might also be a limitation for the validity of the results of all analyzed polymorphisms for other cohorts.

An important constraint of this study is the lack of pharmacokinetic data of atorvastatin and its metabolites, especially of atorvastatin-lactone. Therefore, a future study on non-diabetic patients should include this data and re-analyze the effects of *UGT1A3* and *PON*-locus genetic variability on atorvastatin response and adverse events.

3.4 Conclusions

The hypothesis behind the studies conducted in this thesis was that genetic variability of enzymes involved in the metabolism of atorvastatin might be responsible for adverse events or inferior response in treatment with this drug. This hypothesis was supported by observations of increased atorvastatin-lactone levels in patients suffering from atorvastatin-related myopathy and an increased cytotoxicity of this metabolite compared to the parent drug *in vitro*.

This work showed that UGT1A3 is the major enzyme in the formation of atorvastatin-lactone and that the common variation *UGT1A3*2* is associated with increased lactonization via an augmented expression of this enzyme. In addition, PON1 and PON3 were identified to catalyze atorvastatin-lactone hydrolysis. Genotyping within the *PON*-locus identified *PON1* promoter polymorphisms (*-108T>C, -832G>A, -1741G>A*) and a tightly linked group of *PON3* polymorphisms (*-4984A>G, -4105G>A, -1091A>G, -746C>T* and F21) to be associated with changes in atorvastatin δ-lactone hydrolysis and expression of PON1 but not PON3. This insight into the genotype-phenotype correlations of UGT1A3, PON1 and PON3 provides novel aspects for understanding the metabolism of atorvastatin and for understanding how genetic variation of these loci affects atorvastatin pharmacokinetics. In this way, the basis was prepared for future clinical studies on genetic variability of all important metabolizing enzymes of atorvastatin.

The clinical relevance of genetic variability associated with changes in atorvastatin-lactone levels was tested by retrospective genotyping of two clinical trials. In a study of healthy volunteers, *UGT1A3*2* was associated with a significantly higher AUC of o-OH-atorvastatin-lactone and a higher AUC ratio of lactone to acid of atorvastatin-lactone and o-OH-atorvastatin-lactone. Neither the *UGT1A3*2* variant nor polymorphisms of the *PON*-locus and additionally genotyped polymorphisms *ABCG2c.421C>A, CYP3A4*1B, CYB3A5*3,* were associated with significant changes in the response to atorvastatin in a study with type II diabetes patients on dialysis (i.e. lipid profile or patient survival as analyzed by Kaplan-Meier plots). On the other hand, *UGT1A3*2* was clearly related to prolonged survival in this study regardless of treatment with atorvastatin. This might have possibly resulted from increased levels of the antioxidant bilirubin as a consequence of decreased UGT1A1 due to the *UGT1A1*28* variation, which is linked to the *UGT1A3*2* haplotype.

3.5 Future directions

In today's standard of clinical practice, patients are treated with drugs based on data from the average of the population, although this non-personalized treatment frequently leads to adverse events or non-response. A personalized treatment, as desired by recent efforts worldwide, should take into account the patients' genetic background, such that treatment with certain drugs, to which a patient might not respond or might be prone to develop adverse events could be avoided. This work adds important new aspects to the pharmacogenomics of statin response. It clarified for the first time the role of UGT1A3, PON1 and PON3 in the metabolic pathway of atorvastatin and also the inter-individual variability in expression levels of these enzymes, which are important determinants of exposure to atorvastatin-lactone and therefore possibly related to augmented toxicity (Hermann et al., 2006). Furthermore, these novel findings will be also important for all other substrates of these enzymes. The haplotype *UGT1A3*2* and the SNP *PON1 -108T>C* were found to be important genetic determinants of atorvastatin therapy response as *UGT1A3*2* significantly affected atorvastatin-lactone and o-OH-lactone plasma levels. These genotypes could not predict therapy outcome in diabetes patients on dialysis, possibly due to reasons stated in the discussion above, but they still might be predictive markers of atorvastatin response in non-diabetic cohorts. Future studies should therefore identify the effects of *UGT1A3*2* and *PON*-locus polymorphisms in non-diabetic patients, with a focus on the pharmacokinetic data of atorvastatin and its metabolites.

In addition, UGT1A3 and the paraoxonases are not only involved in the metabolism of atorvastatin, but observed differences in the expression of these enzymes due to *UGT1A3*2* and *PON*1* haplotypes should affect the metabolism of their xenobiotic or endogenous substrates. A good example was shown in section 2.2.5.2, which clarified that *UGT1A3*2* significantly affects the glucuronidation of the important bile acids or bile acid precursors deoxycholic- and lithocholic-acid, as well as, of estrone. Other drugs with known involvement of UGT1A3 in their metabolism are the statin pitavastatin (Fujino et al., 2003), the anti-HIV maturation inhibitor bevirimat (Wen et al., 2007), the major buprenorphine metabolite nor-buprenorphine (Rouguieg et al., 2009) and a DPP-4 inhibitor for the treatment of type II diabetes, which showed a rare glucuronidation reaction at a carbamic acid (Gunduz et al., 2009). This leaves these drugs promising candidates for future studies on the predictive effect of the *UGT1A3*2* haplotype on therapy response.

Furthermore, this work contributed to a better understanding of the mechanism, by which PON1 promoter polymorphisms affects the expression of this enzyme. As a consequence, these changes in expression should affect all or most other substrates of PON1. Paraoxonases are known to catalyze the hydrolysis of lactones. In addition to a wide variety of endo- and xenobiotics, PON1 and PON3 have been reported to hydrolyze the statins mevastatin, simvastatin and lovastatin, as well as, the potassium-sparing diuretics, spironolactone and canrenone (Draganov et al., 2005, Billecke et al., 2000). Paraoxonases also possess organophosphatase activity and are able to hydrolyze insecticides (parathion, diazinon and chlorpyrifos; Costa et al., 2003) and nerve agents (sarin, soman; Billecke et al., 2000). In addition, carriers of the low expression phenotype might also be more sensitive to pesticides, a fact which has recently been shown for the low (organophosphatase) activity genotypes *PON1*-Q192R and -L55M (Singh et al., 2011).

PON1 was also reported to prevent LDL oxidation (Mackness et al., 1991) because of its ability to convert multi-oxidized phospholipids, which after oxidative fragmentation produce molecules that are able to induce monocyte-endothelial interactions (Watson et al., 1995) involved in the formation of atherosclerotic lesions. PON3 was also shown to have antioxidant properties (Reddy et al., 2001). Draganov et al., 2000 observed that rabbit PON3 could inhibit copper induced LDL oxidation 100 times more effectively as compared to rabbit PON1. Hence genetic variability within the *PON*-locus may also affect the susceptibility for atherosclerosis and coronary heart diseases (Sozmen; Bourquard: in Mackness et al., 2008).

In conclusion, *UGT1A3*2* and *PON1 -108T>C* significantly affect the expression levels of UGT1A3 and PON1, respectively. This was shown by significantly increased UGT1A3 mRNA and protein expression, as well as, atorvastatin-lactonization in *UGT1A3*2* carriers and significantly decreased PON1 mRNA and protein expression, in addition to, atorvastatin-lactone hydrolysis in *PON1 -108T* carriers. Consequently, these variations are associated with higher levels of atorvastatin-lactone, a metabolite associated with toxicity. Although no associations were found with changes in statin response in diabetes patients, these genetic variations may influence response to therapy or adverse events in statin-treatment in other patient cohorts. Additionally, these genetic variants might also affect drugs with a significant metabolic involvement of UGT1A3 or PON1 including anti-diabetic, anti-HIV, analgesic and diuretic drugs.

4. Methods

4.1 Chemical reagents

Atorvastatin calcium salt, atorvastatin-d5 sodium salt, atorvastatin lactone, atorvastatin-d5 lactone, lovastatin, lovastatin-d3, lovastatin hydroxy acid sodium salt, lovastatin-d3 hydroxy acid sodium salt, pravastatin lactone and pravastatin lactone-d3, simvastatin-d6, simvastatin hydroxyl acid ammonium salt, simvastatin-d6 hydroxy acid ammonium salt, rosuvastatin calcium salt, rosuvastatin-d6 sodium salt, rosuvastatin lactone, rosuvastatin lactone-d6 were purchased from Toronto Research Chemicals Inc. (North York, Canada). All other chemicals were obtained at highest available grade from Sigma-Aldrich (St. Louis, USA).

4.2 Equipment

mass spectrometrical analytics:

Esquire HCT ultra ion trap mass spectrometer	Bruker Daltonics
HPLC 1100-System with binary pump G1312A, degasser G1379A, well-plate sampler G1367A and column thermostat G1330B	Agilent
LC-MSD system HP Series 1100 with binary pump, degasser, autosampler and mass selective detector equipped with an electrospray ion source	Agilent
6460 triple quadrupole mass spectrometer 1200 HPLC system consisting of degasser G1379B, binary pump G1312B, well-plate sampler G1367D and column thermostat G1316B,	Agilent

columns:

Shield RP18 3.5µm + pre-column Security Guard C-8	XBridge
LUNA 5µ C8(2) 2x150mm	Phenomenex
ZORBAX SB-C18 Rapid Resolution HD 2.1x50mm 1.8 Micron	Agilent

centrifuges:

Biofuge pico	Heraeus
centrifuge 5417C	Eppendorf
Hettich Universal 32	Hettich
ThermoMegafuge	Heraeus
ultracentrifuge L5-65	Beckman

software:

Geneious, 4.8.5; Biomatters Ltd. (Auckland, New Zealand)

GraphPad Prism, 4.0; GraphPad Software (San Diego, USA)

R, 2.1

SPSS 16.0.2 (SPSS, Munich, Germany)

PHASE 2.1.1

Haploview 4.1

other equipment:

6-well plates, tissue culture coated	Greiner
2100 Bioanalyzer	Agilent
3500 DX, capillary sequencer	Applied Biosystems
7900 HT Real Time PCR System (Taq Man)	Applied Biosystems
Bio-Rad protein assay, BSA standard protein	BioRad
cell culture flasks, polystyrene; T175, T-75, T-25	Corning
cuvettes for single usage	Sarstedt
DNA 1000 LabChipKit, RNA 6000 Nano LabChipKit	Agilent
DNA Sep Column System	Transgenomic
fast-blot gadget	Biometra
fastprep-24 homogenizer	MP-Biomedicals
fastprep-tubes containing Lysis Matrix	MP-Biomedicals
just-spin gel extraction columns	Genaxxon
millipore-device Milli Q	Millipore
nanodispenser robot	Sequenom
nanodrop 2000c	Thermo Scientific
nitrocellulose transfer membrane, 0.45 µm pore-size	Whatman
odyssey infrared imaging system	LI-COR Biosc.
petri-dishes, 94mm diameter	Greiner
petri-dishes, tissue culture coated, 94mm diameter	Greiner
pH-Meter CG840	Schott
pocketbloc thermomixer	Biozym
PTC-200 PCR machine	BioRad
puredisk robot for pipetting	Cybio
SDS-PAGE chamber Protean II xi cell	Bio-Rad
Sequenom MALDI-TOF mass-spectrometer	Bruker Sequenom
shaker (384-well plates)	Heidolph
shaker (western-blots)	GFL

thermal cycler DYAD PCR (Tetrad PTC-200/225)	BioRad
thermal cycler PTC-200 PCR	BioRad
vortexer	Heidolph
water bath var3185 Assistant	WTE
WAVE DNA Fragment Analysis System (DHPLC)	Transgenomic

4.3 Buffers and solutions

Buffer for cell-homogenisation	EDTA	1 mM
(freshly prepared)	DTT	1 mM
	Pefa Bloc	0.2 mM
	HEPES pH 7,4	10 mM
	KCl	0.15 M
Resuspension buffer for microsomes	sodium phosphate buffer pH 7.4	0.1 M
Washing buffer	EDTA	1 mM
for microsomes	$Na_4P_2O_7$ pH 7.5	0.1 M
TAE (50 x)	glacial acetic acid	57.1 ml
	EDTA (0.5 M, pH 8)	100 ml
	$H_20_{millipore}$	ad 1000 ml
DNA loading buffer (5 x)	ficoll (20 %)	874 µl
	bromophenolblue (0.5 %)	87.4 µl
	EDTA (0.5 M, pH 8)	38 µl
Laemmli sample buffer (5 x)	tris-HCl pH 6.8	306 mM
(Ausubel, 2002)	β-mercaptoethanol	25 %
	SDS	10 %
	bromophenolblue	0.1 %
	$H_20_{millipore}$	ad 75 ml
	glycerin	ad 100 ml

Electrophoresis buffer (10 x)	tris base	150 g
(SDS-PAGE)	glycine	720 g
	SDS (20 %)	250 ml
	$H_20_{millipore}$	ad 5000 ml
Blotting buffer	tris base	29 g
	glycine	14.6 g
	SDS 20 %	9.25 ml
	methanol	1000 ml
	$H_20_{millipore}$	ad 5000 ml
APS 10 %	ammoniumpersulfate	1 g
	$H_20_{millipore}$	ad 10 ml
TBS (10 x)	tris base	150 g
	NaCl	400 g
	KCl	10 g
	$H_20_{millipore}$	ad 5000 ml
TBST (1 x)	TBS (10 x)	500 ml
	tween 20 (50 %)	10 ml
Tris-HCl (1.5 M), pH 8.8	tris base	90.75 g
	pH adjusted to 8.8 by HCl	
	$H_20_{millipore}$	ad 500 ml
Tris-HCl (0.5 M), pH6.8	tris base	30 g
	pH adjusted to 6.8 by HCl	
	$H_20_{millipore}$	ad 500 ml
Ampicillin	100 mg/ml	100 µg/ml
	(in 50 % EtOH)	

Tris-HCl 50 mM, pH 8.8	tris base	302.9	mg
	pH adjusted to 8.8 by HCl		
	$H_20_{millipore}$	ad 50	ml
NADPH regenerating system	glucose-6-P-dehydrogenase	20	U
	$MgCl_2$	50	mM
	$NADP^+$	5	mM
	glucose-6-phosphate	40	mM
	0.1 M sodium phosphate buffer pH 7.4	390	µl

4.4 Liver samples

Liver tissue and corresponding blood samples were collected in a previous study at the Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany. Samples were derived from patients undergoing liver surgery at the Campus Virchow, Humboldt University, Berlin, Germany. All tissue samples were examined by a pathologist and only histologically nontumorous tissue was used. Clinical patient documentation for all samples included age, sex, medical diagnosis, presurgical medication, liver function parameters, and alcohol and smoking habits. Patients with hepatitis, cirrhosis or chronic alcohol use were excluded. Finally 150 liver samples, from which high quality RNA and complete documentation could be obtained were used. The preparation of human liver microsomes had been described before (Lang et al., 2001).

4.5 Genetic and cellbiologic methods

4.5.1 Plasmids and expression vectors

Plasmids were generated based on the sequencing vector pCR4 TOPO (Invitrogen, Carlsbad, CA), and the expression vectors pCMV4 (U.M. Zanger, IKP Stuttgart) and pCR3.1/V5-HisTOPO (Invitrogen, Carlsbad, CA). Desired fragments were amplified from human liver cDNA with primers listed in table 13 (Positions according to *UGT1A3*; NM_019093, *UGT1A4*; NM_007120 and *PON1*; NM_000446.5, and *PON3*; NM_000940.2) using the Expand High Fidelity PCR System (Roche, Mannheim) according to manufacturer's instructions. Annealing temperatures were optimized for each fragment. Correct amplification was tested on an agarose-gel. Fragments were excised from the gel, purified by applying JustSpin Gel Extraction columns (Genaxxon BioScience, Ulm) and used for TA-cloning in

pcDNA4 TOPO or pcDNA3.1/V5-His TOPO as proposed by the producer (Invitrogen, Carlsbad, CA).

Table 13: primers used for cDNA amplification via PCR and control sequencing of plasmids and expression vectors.

Name	cDNA Position	Primer sequence (5'→ 3')	Ampli-fication product (bp)	Purpose
1A3_fragment_f	347-371	TGTTGAACAATATGTCTTTGGTCTA	698	PCR, sequencing
1A4_fragment_f	258-278	GAAGGAATTTGATCGCGTTAC	787	PCR, sequencing
1A5_fragment_f	171-189	GGTGGTGGTCCTCACCCTG	874	PCR
1A_fragment_r	1024-1044	GTTCGCAAGATTCGATGGTCG		PCR, sequencing
1A3_komplett_f	-65 to -44; -53 mut .C	AAGCTTGAAGAAAGCAAACGTAGCAGGC	1704	PCR
1A3_komplett_r	1606-1627	TCTAGATACCTTATTTCCCACCCACTTC		PCR
PON3_komplett_f	1-16	AGATCTAGTCGCCGCTGGGCAC	1203	PCR, sequencing
PON3_komplett_r	1170-1193	AAGCTTTTGGTGTTTGCTATTTACTTAC		PCR, sequencing
PON1_kompl_neu_f	95-115	ACCATGGCGAAGCTGATTGCG	1239	PCR, sequencing
PON1_kompl_neu_r	1306-1327	GAATTCTACACATCATATCACTCCCAGT		PCR, sequencing
UGT1A3_for	449-468	TAACAGACCCCGTTAACCTC		sequencing
UGT1A3_rev	1151-1169	ACCATGGGAACGCCATTGC		sequencing
M13_for (pCR4 TOPO)	388-405	TTGTAAAACGACGGCCAGT		sequencing
M13_rev (pCR4 TOPO)	205-221	CAGGAAACAGCTATGAC		sequencing
PON1_seq1_f	136-156	ACTGGCACTCTTCAGGAACCA		sequencing
PON1_seq1_r	696-716	AGACAACATACGACCACGCTA		sequencing
PON1_seq2_f	600-620	ATGATATTGTTGCTGTGGGAC		sequencing
PON1_seq2_r	1278-1298	TGCTTTGATGCTTCATGATGT		sequencing
PON3_seq1_f	24-43	CCATGGGGAAGCTCGTGGCG		sequencing
PON3_seq1_r	653-674	TGGCCACCACTTTAACCTCCCT		sequencing
PON3_seq2_f	520-545	TGTGAATGACATTGTGGTTCTTGGAC		sequencing
PON3_seq2_r	1070-1095	AGAGTCTAGAGCTCACAGTACAGAGT		sequencing

Cloning of *UGT1A3* into the mammalian expression vector pCMV (U.M. Zanger, IKP Stuttgart) was performed by digesting with *Hind*III and *Xba*I resulting in the plasmids

pSRM12 and pSRM13. Cloning of *PON3* from pSRM18/19 into the mammalian expression vector pCR3.1/V5-HisTOPO/lacZ (Invitrogen, Carlsbad, CA) was done using digestion by *Hind*III and *Not*I. Fragments were ligated with T4 DNA Ligase (Roche, Mannheim) and applied for transformation of chemically competent TOP10 *E.coli* K12 (Invitrogen, Carlsbad, CA) following manufacturer's instructions.

Transformants of TA cloning reactions and cloning of ligations were selected on LB agar containing 100µg/ml ampicillin. A subset of clones were selected, amplified in a volume of 2ml and used for plasmid preparation applying Qiagen MiniPrep (Qiagen, Hilden). Concentrations of plasmids were determined using the Nanodrop2000c device. Control digestions were performed:

			size of control fragment:
UGT1A3-fragment:		*EcoR*I	675bp
UGT1A4-fragment:		*EcoR*I/*Xba*I	726bp
UGT1A3:		*Hind*III/*Xba*I	1704bp
		*Xba*I/*Sac*II (for SNP W11R)	1606bp
PON1:	pSRM22/23/24:	*BamH*I	862bp
PON3:	pSRM18/19:	*EcoR*I	1227bp
	pSRM20/21:	*Hind*III	1221bp

Candidate clones after control digestions were selected for sequencing. Sequencing of plasmids was performed using 200-300ng plasmid-DNA, 5.12pmol primer (table 13**;** Position according to NM_019093; UGT1A3, NM_007120; UGT1A4 and NM_000446.5; PON1 and NM_000940.2; PON3), BigDye Terminator v3.1 Mix and Big Dye Seq Buffer per reaction. Cycle sequencing PCR was done at 96°C for 1min followed by 25 cycles of 96°C for 10sec, annealing at 50°C for 10sec and amplification at 60°C for 4min. Samples were purified with Sephadex matrix (G50, Amersham, Munich) diluted 4-fold and analyzed by capillary sequencing on an ABI3500DX (Applied Biosystems Carlsbad, CA).

Clones containing plasmids with verified desired sequence were amplified in 50ml volume. Plasmid DNA was prepared using the PureYield Plasmid Midiprep System (Promega, Madison, WI, USA). Plasmids and expression vectors are shown in figure 34.

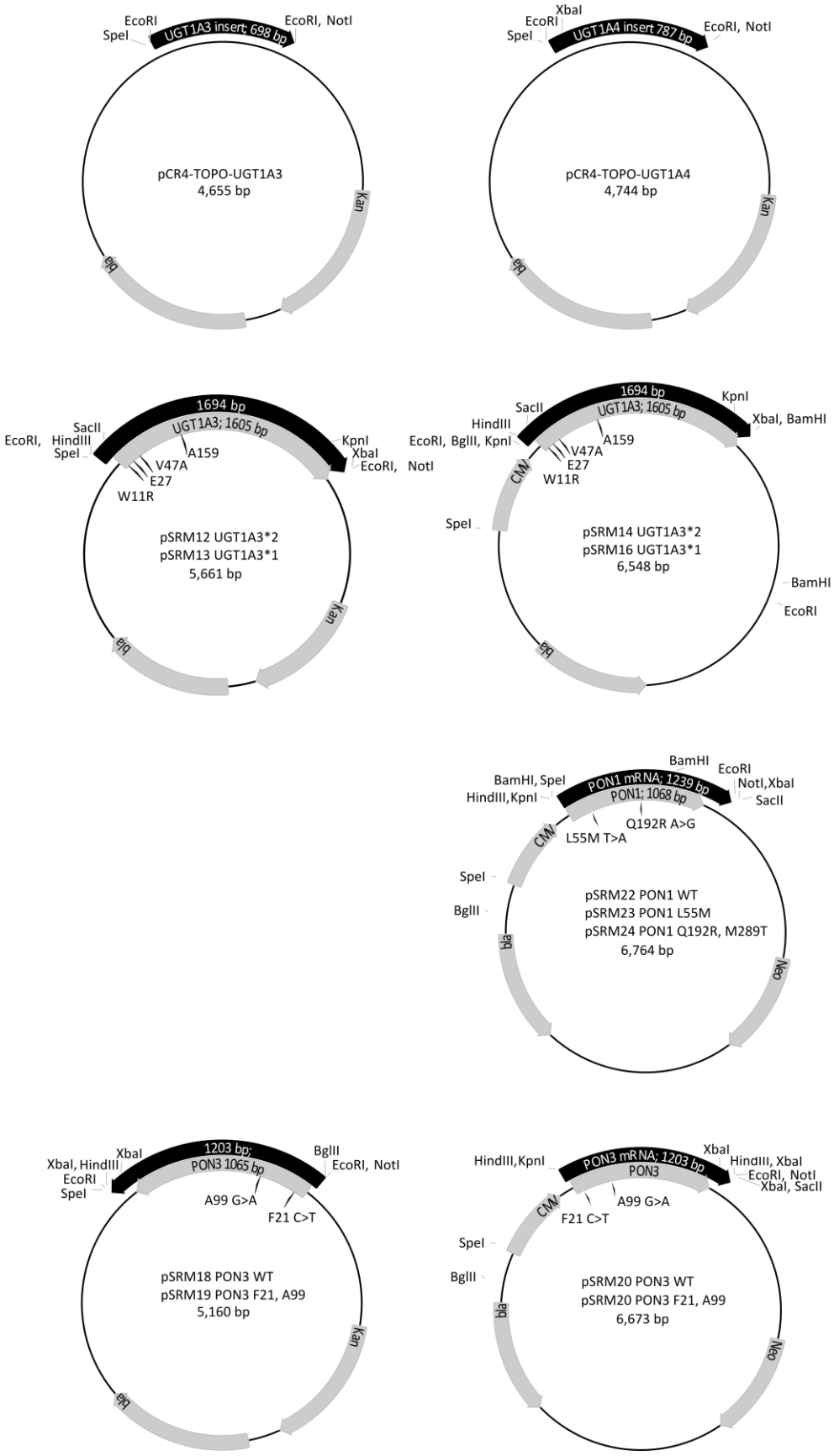


Figure 34: Plasmids and expression vectors used as qPCR standard and for recombinant protein expression. Inserted regions are represented by black arrows, whereas genes and CMV promoter are marked in grey. Vectors are based on pCR4 TOPO (pCR4-TOPO-UGT1A3 & UGT1A4, pSRM12 & 13, pSRM18), pCR3.1/V5-His TOPO (pSRM22-24) and pCMV4 (pSRM20 & 21).

4.5.2 Cell culture and transient expression of recombinant proteins

A **monkey derived kidney cellline (COS-1)** was used for the transient recombinant expression of proteins. COS-1 cells were cultured in DMEM medium supplemented with 10 % fetal bovine serum, 1% penicillin/ streptomycin and 1% L-glutamine. Cells were cultured in an incubator at 37 °C in 5% CO_2 atmosphere in polystyrene cell culture flasks (Corning, New York). On reaching confluence, cells were detached using 0.25% trypsin-EDTA solution and seeded again.

One day before the transfection, COS-1 cells were seeded in 6-well plates (3-4 x 10^5 cells per well, Nunc GmbH, Wiesbaden) and 2ml medium or tissue culture coated 10cm petri-dishes (1.8 x 10^6 cells per petri-dish) and 10ml medium. Cells were transiently transfected using Lipofectamine 2000 according to the manufacturer's instructions. One hour before transfection, culture medium was replaced by antibiotics and serum-free Opti-MEM medium. For test transfections a mixture of 3µg of the respective expression plasmid, 900ng pCMV β (Clontech) and Lipofectamine 2000 (2µl/µg plasmid) was added to each well and incubated at 37 °C. Five hours after the transfection, the DNA-liposome complex was removed and replaced by pre-warmed culture medium. For large scale transfections in petri-dish format 4 µg of plasmid were transfected and incubated at 37 °C. After 48 hours, cells were washed twice with cold phosphate-buffered saline (PBS) and harvested in 200µl (6-well plate) or 500µl (petri-dish) sodium phosphate buffer (0.1M) containing 250mM sucrose. Preparation of microsomal fractions was performed according to IKP SOP MO-M-006. Cells were homogenized with lysing-matrix D in the Fast Prep System (both from MP-Biomedicals, Illkirch, France), centrifuged and dissolved in 2ml sodium phosphate buffer (0.1M) containing 250mM sucrose. Samples were centrifuged at 10,000 x g at 4°C for 20min, the supernatant (S9) was centrifuged again at 100,000 x g at 4°C for 60min and the pellet homogenized in 0.1M sodium phosphate buffer (0.1M) containing 10% glycerol. Resulting microsomal fractions were frozen in aliquots of 25µl and stored at -80°C.

4.6 Quantitative methods

4.6.1 Quantification of total nucleic acid content

Concentrations of nucleic acid samples were determined with a Nanodrop 2000c (Thermo Scientific). Additionally, RNA integrity and quantity was analyzed on the Agilent 2100 bioanalyzer using the RNA 6000 Nano Kit. Preliminary quantification of *PON3 wild-type* and *splice-variant* PCR products from cDNA was performed using the DNA 7500 Kit.

4.6.2 Quantification of mRNA expression

Total RNA was prepared from liver tissue by Trizol (Invitrogen, Paisley, USA) extraction and using the RNeasy Mini Kit with on-column DNase treatment (Qiagen, Hilden, Germany) as described previously (Gomes et al., 2009). Only high quality RNA preparations according to Agilent Bioanalyzer (Nano-Lab Chip Kit, Agilent Technologies, Waldbronn, Germany) RIN assignment (>7) were used in this study. Expression levels of mRNA transcripts were assessed by the Human-6 v3 Expression BeadChips (Illumina, Eindhoven, Netherlands) and performed by Microarray facility Tübingen. Quantil normalized expression data of the 15 candidate esterases was used in the analysis.

For PCR based quantification, synthesis of cDNA was performed with 1µg total RNA using the TaqMan Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) using random hexamers according to the supplier's instructions. Quantification of *PON1*, *PON3 wild-type*, *PON3 splice-variant* and *UGT1A3* mRNA was performed by specific TaqMan real-time PCR on a 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA) using exon-exon boundary spanning primers (400nM each) and FAM-labeled MGB probes (200nM) (see table 14**;** Position according to cDNA *UGT1A3*; CCDS2509.1, *PON1*; J01636.1 and *PON3;* NM_000940.2;). Specificity for *UGT1A3* was confirmed using DNA plasmids constructed with *UGT1A3* and *UGT1A4* genomic fragments (pCR4-TOPO-UGT1A3 and pCR4-TOPO-UGT1A4; see figure 34), which were EcoRI digested to yield linear DNA as UGT1A3 Taqman standard (10fg/µl to 1pg/µl) and as UGT1A4 negative control (1 to 100pg/µl). Specificity for *PON1* was confirmed using DNA plasmids constructed from *PON1* and *PON3 wild-type* complete cDNA (*PON1*: pSRM22 and *PON3*: pSRM18), which were *Hind*III+*Xba*I digested to yield linear DNA as *PON1* Taqman standard (10fg/µl to 1pg/µl) and *Eco*RI digested to gain a *PON3 wild-type* negative control (1 to 100pg/µl). Specificity for *PON3 wild-type* and *splice-variant* were confirmed using DNA plasmids constructed from *PON3 wild-type* and *splice-variant* complete cDNA (*PON3 wild-type*: pSRM18 and *PON3 splice-variant*: pSRM17), which were *Eco*RI digested to yield linear DNA as pSRM18 and pSRM19 Taqman standard (10fg/µl to 1pg/µl) and as negative control vice versa (1 to 100pg/µl). PCR was performed using 2x universal PCR Master Mix (Applied Biosystems) in a final volume of 12.5µl and the following cycling conditions: 50°C for 2min; 95°C for 10min followed by 40 cycles of 95°C for 15sec and 60°C for 1min. Results were normalized to RPLP0 mRNA levels and expressed in relation to the lowest value, which was set at 1.0.

Table 14: Amplification primers used for quantitative PCR.

Name	cDNA Position	Primer sequence (5'→ 3')	Amplification product (bp)	Purpose
1A3_tq_neu_f	738-763	GGATATTCTCAGTCATGCATCTGTGT	297	for primer
1A3_tq_neu_r	1017-1034	TTCGATGGTCGGGTTCCA		rev primer
TQ_PON3_for	181-209	TGAAGATATTGATATACTTCCTAGTGGGC	wt: 136 var: 132	for primer
TQ_PON3_WT_rev	297-316	TGCCCTTGGGTTTTGTTCAT		rev primer
TQ_PON3_VAR_rev	67-86(Exon splVar)	TATTTGCCGTTCTGCAGCCT		rev primer
TQ_UGT1A3	795-807	6FAM-CCCCAGGCCAATC-MGB		probe
TQ_PON3_WT	218-234	6FAM-ATCTCCAGTGGATTAAA-MGB		probe
TQ_PON3_VAR	218-8(Exon splVar)	6FAM-ATCTCCAGTCTGCAGGT-MGB		probe
TQ-lacZ	1412-1432	6FAM-CCGCACCAATCGCCCTTCCCA-MGB		probe
PON1_Hs00166557_m1	1006	TCCTGCATCAGAGGTGCTTCGAATC	122	mix
RPLP0_4326314E	exon3	endogenous control, VIC / MGB probe	105	mix

4.6.3 Quantification of total protein content

Protein concentration of samples was determined by the method of Bradford according to IKP SOP MO-M-007. In short, 5µl of the sample were diluted with 795µl water. After the addition of 200µl Bradford protein reagent (BioRad) and an incubation time of 10min, optical density was determined at 595nm. Quantification was performed in comparison to a bovine serum albumine standard (1.4 to 8.4µg of protein).

4.6.4 Quantification of proteins by SDS-PAGE and immunoblotting

Relative protein quantification was performed by western blot analysis. Electrophoretic separation of samples in Laemmli-buffer was done on a 10% SDS-polyacrylamidgel (170V, 3h to 4h).

Table 14: Composition of a 10% SDS-polyacrylamidgel.

	stacking gel	resolving gel
H2O	12 ml	6,1 ml
Acrylamid/BIS (30:0,8)	10 ml	1,35 ml
1,5 M Tris pH 8,8	7,5 ml	-
0,5 M Tris pH 6,8	-	2,5 ml
10 % SDS	300 µl	100 µl
TEMED	30 µl	10 µl
10 % APS	300 µl	100 µl

Proteins were transferred by semidry-blotting onto a nitrocellulose membrane (3mA/cm^2, 15min). Membranes were blocked with 5% skim milk in TBST for 1h at room temperature. Primary antibodies were diluted as described (see table 15) in 1% skim milk-TBST and blots were incubated for 1h at room temperature or over night at 8°C in this solution. Membranes were washed and incubated (under protection from light) for 30min at room temperature with the corresponding secondary antibody (see table 15). Finally membranes were washed again and the ODYSSEY infrared imaging system (LI-COR) was used for detection. Relative quantification was performed based on standard curves of recombinant human UGT1A1, UGT1A3 or pooled human liver micosomes (for PON1 and PON3) recorded on each blot. Results were expressed in relation to the lowest value set at 1.0. For molecular weight estimation high range rainbow marker (14.3 to 220kDa; Amersham Biosciences) was used. Deglycosylation of samples was performed according to the manufacturer's protocol by incubation with endoglycosidase PNGase F (New England Biolabs, Ipswich, USA) for 1h at 37°C after denaturation at 100°C for 10min. For quantification of microsomal UGTs the combined intensity of stained bands in untreated (glycosylated) samples was used.
For recombinant expression experiments, transfection efficiency was tested and normalization performed by co-staining against Neo-P-transferase 2 (Neomycin resistance gene on pCR3.1/V5-HisTOPO (Invitrogen, Carlsbad, CA) based plasmids) or against β-Galactosidase (co-transfected plasmid pCMV-β (Oliver Burk, IKP Stuttgart).

Table 15: Antibodies for immunoblotting and applied dilutions of primary and secondary antibodies prepared in 1% skim milk-TBST.

immunogen	**β-Gal**	**PON1**	**PON3**	**Neo-P-Transferase 2**	**UGT1A1**	**UGT1A3**
size of protein	116kDa	38 & 40kDa	38kDa	30kDa	54kDa	54 & 57kDa
description	mouse monoclonal	mouse monoclonal	mouse monoclonal	mouse monoclonal	rabbit polyclonal	mouse monoclonal
antibody	MBL M094-3	sc-59646	ab71994	ab60018	WB-UGT1A1	ab57400
company	MoBiTec	santa cruz	abcam	abcam	BDBiosciences	abcam
dilution	1:1000	1:1000	1:2000	1:1000	1:500	1:1000
sec. antibody	IRDye800 (1:10000)	IRDye800 (1:10000)	IRDye800 (1:10000)	IRDye800 (1:10000)	IRDye680 (1:10000)	IRDye800 (1:10000)

4.6.5 Mass-spectrometric based quantification

4.6.5.1 Quantification of atorvastatin, atorvastatin-lactone and their hydroxyl-metabolites

Atorvastatin-hydroxylation was determined with 5pmol recombinantly expressed cytochrome P450 (CYP) enzymes (BD Biosciences) with 10 and 100µM atorvastatin and 10µl of the NADPH regenerating system (20U glucose-6-P-dehydrogenase, 50mM $MgCl_2$, 5mM $NADP^+$, 40mM glucose-6-phosphate) in a volume of 100µl 0.1M sodium phosphate buffer (pH 7.4). Reactions were stopped after 10min incubation time at 37°C by the addition of 50µl cold acetonitril containing 250mM formic acid and 10µM deuterated standard for atorvastatin and each hydroxyl-metabolite.

Atorvastatin-lactone formation by human liver microsomes and recombinant UGT Supersomes (BD Biosciences) was measured by incubating 25µg of protein in 50mM Tris HCl, pH 7.4, with 5mM $MgCl_2$, 25µg/ml alamethicin and 10µM atorvastatin (solub. in acetonitril/H_2O) in a total volume of 100µl. Following pre-incubation for 10min at 37°C the reaction was started by the addition of 10mM UDP-glucuronic acid. Similar conditions were applied for lactone formation from 10µM pravastatin, rosuvastatin, simvastatin- and lovastatin-acid.

Atorvastatin-lactone hydrolysis was determined in human liver microsomes. Incubation time, substrate concentration and protein content were chosen with care to guarantee measurements within the linear range for each condition. Separate incubation experiments were always carried out together with a denaturated sample of pooled human liver microsomes to guarantee reproducibility and to subtract non-enzymatic conversion. Incubations were performed with 5µg of protein in 50mM Tris HCl, pH 7.4 and 10mM $CaCl_2$ in a total volume of 100µl. The reaction was started by adding 10µM atorvastatin lactone (solub.in acetonitrile). Lactone formation and hydrolysis reactions were stopped after 30min by adding 100µl ice-cold 250mM formic acid/acetonitrile and immediate cooling on ice. Lovastatin hydrolysis was determined under similar conditions using 100µg pooled HLMs and 50µM lovastatin and an incubation time of 60min for the performed preliminary experiment. After adding 10µM deuterated standard of the corresponding statin-acid or lactone, samples were centrifuged and analyzed by LC-MS-MS. HPLC separation conditions, MS parameters, as well as, precursor and product ions (m/z) for atorvastatin and its metabolites, for pravastatin and rosuvastatin, acid and lactone, are listed in table 16 and for lovastatin and simvastatin, lactone and acid are listed in table 17. Possible quantification ranged from 0.5 to 500pmol per sample.

4.6.5.2 Quantification of estrone-glucuronide

Estrone-glucuronidation by human liver microsomes and recombinant UGT Supersomes (BD Biosciences) was measured by incubating 50µg of protein in 50mM Tris HCl, pH7.4, with 5mM $MgCl_2$, 25µg/ml alamethicin and 100 or 10µM estrone in a total volume of 100µl. Following pre-incubation for 10min at 37°C the reaction was started by the addition of 10mM UDP-glucuronic acid. The reaction was stopped after 30min by adding 100µl ice-cold acetonitrile with 642nM internal standard (Trimegestone-glucuronide) and immediate cooling on ice. Samples were centrifuged, diluted 1:1 in 0.1% acetic acid and analyzed by LC-MS. HPLC separation conditions, MS parameters, as well as, precursor and product ions (m/z) are listed in table 18. Possible quantification ranged from 1 to 1000pmol per sample.

4.6.5.3 Quantification of DCA- and LCA-glucuronide

3.4 to 6.8µg protein of human liver microsomes and recombinant UGT Supersomes (BD Biosciences) was placed into 96-well plates and frozen until the day of the experiment. DCA-/LCA-glucuronidation was measured by adding 50mM Tris HCl, pH7.4, with 5mM $MgCl_2$, 25µg/ml alamethicin, 10mM UDP-glucuronic acid and 20 or 2µM DCA (solub. in acetonitrile/H_2O) or LCA (solub. in acetonitrile) in a total volume of 100µl and incubating the reaction at 30°C in a PCR machine. The reaction was stopped after 30min by cooling to 8°C and adding 100µl ice-cold acetonitrile with 1.76µM internal standard (ursodeoxycholic acid acyl-beta-D-glucuronide). Samples were centrifuged, diluted 1:1 in 0.1% acetic acid and analyzed by LC-MS-MS. HPLC separation conditions, as well as, MS parameters, precursor and product ions (m/z) are listed in table 18. Relative quantification ranged from glucuronide produced in 0.1 to 100µg pooled liver microsomes under conditions mentioned above.

Table 16: Liquid chromatography and mass spectrometry conditions applied in the quantification of atorvastatin, atorvastatin-lactone and their hydroxyl metabolites, and of the acid and lactone form of pravastatin and rosuvastatin.

	atorvastatin + metabolites	**pravastatin + lactone**	**rosuvastatin + lactone**
Machine	Esquire HCT ultra ion trap mass spectrometer (Bruker Daltonics)	Esquire HCT ultra ion trap mass spectrometer (Bruker Daltonics)	Esquire HCT ultra ion trap mass spectrometer (Bruker Daltonics)
LC-Parameters			
Injection Volume (µl)	10	10	10
Column	XBridge Shield RP18 3.5µm + pre-column Security Guard C-8	XBridge Shield RP18 3.5µm + pre-column Security Guard C-8	XBridge Shield RP18 3.5µm + pre-column Security Guard C-8
Solvents	A: 1 mM formic acid B: acetonitrile	A: 1 mM formic acid B: acetonitrile	A: 1 mM formic acid B: acetonitrile
Flow (ml/min)	0.3	0.4	0.4
Temperature	30	30	30
Pump-Timetable	time (min) B (%)	time (min) B (%)	time (min) B (%)
	0 37	0 25	0 37
	4 40	1 25	1 37
	13 40	10 70	8 80
	15.5 45	10.5 70	10 80
	16.5 45	14 25	10.5 37
	16.8 37		14 37
	20 37		
MS-Parameters			
Gas Temp (°C)	300	300	300
Gas Flow (l/min)	10	10	10
Nebulizer (psi)	45	45	45
Sheath Gas Temp. (°C)			
Sheath Gas Flow (l/min)			
Capillary (V)	4100	4500	4500
Nozzle Voltage (V)			
Polarity	negative	positive	positive
Mode	MRM	MRM	MRM
Compounds	Ions Mass; EIC	Ions Mass; EIC	Ions Mass; EIC
	p-OH-ATV 575; 440.2, 466.2	PRV 424; 447.1	ROV 481; 482.1
	d5-p-OH-ATV 580; 445.2, 471.2	d3-PRV 427; 450.1	d6-ROV 487; 488.1
	p-OH-ATV-L 557; 448.4	PRV-L 406; 429.1	ROV-L 464; 464.1
	d5-p-OH-ATV-L 562; 448.4	d3-PRV-L 409; 432.1	d6-ROV-L 470; 470.1
	o-OH-ATV 575; 466.2		
	d5-o-OH-ATV 580; 471.2		
	o-OH-ATV-L 557; 448.4		
	d5-o-OH-ATV-L 562; 448.4		
	ATV 559; 440.2, 466.2		
	d5-ATV 564; 445.2, 471.2		
	ATV-L 541.2; 448.2		
	d5-ATV-L 546.2; 453.2		

Table 17: Liquid chromatography and mass spectrometry conditions applied in the quantification of lovastatin and simvastatin.

	lovastatin + acid		simvastatin + acid	
Machine	Esquire HCT ultra ion trap mass spectrometer (Bruker Daltonics)		Esquire HCT ultra ion trap mass spectrometer (Bruker Daltonics)	
LC-Parameters				
Injection Volume (µl)	10		10	
Column	XBridge Shield RP18 3.5µm + pre-column Security Guard C-8		XBridge Shield RP18 3.5µm + pre-column Security Guard C-8	
Solvents	A: 1 mM formic acid B: acetonitrile		A: 1 mM formic acid B: acetonitrile	
Flow (ml/min)	0.4		0.4	
Temperature	30		30	
Pump-Timetable	time (min)	B (%)	time (min)	B (%)
	0	45	0	45
	1	45	1	45
	8	80	8	80
	10	80	10	80
	10.5	45	10.5	45
	14	45	14	45
MS-Parameters				
Gas Temp (°C)	300		300	
Gas Flow (l/min)	10		10	
Nebulizer (psi)	45		45	
Sheath Gas Temp. (°C)				
Sheath Gas Flow (l/min)				
Capillary (V)	4500		4500	
Nozzle Voltage (V)				
Polarity	positive		positive	
Mode	MRM		MRM	
Compounds	Ions	Mass; EIC	Ions	Mass; EIC
	LOV-A	422; 445.1	SIM-A	436; 459.1
	d3-LOV-A	425; 448.1	d6-SIM-A	442; 465.1
	LOV-L	404; 427.1	SIM-L	418; 441.1
	d3-LOV-L	407; 430.1	d6-SIM-L	424; 447.1

Table 18: Liquid chromatography and mass spectrometry conditions applied in the quantification of estrone, deoxycholic-acid, lithocholic-acid.

	estrone glucuronide		deoxycholic-acid glucuronide		lithocholic-acid glucuronide	
Machine	MSD		QQQ		QQQ	
LC-Parameters						
Injection Volume (µl)	20		20		20	
Column	Phenomenex LUNA 5µ C8(2) 2x150mm		Agilent ZORBAX SB-C18 Rapid Resolution HD 2.1x50mm 1.8 Micron		Agilent ZORBAX SB-C18 Rapid Resolution HD 2.1x50mm 1.8 Micron	
Solvents	A: H_2O+0.1% acetic acid B: acetonitrile		A: H_2O+0.1% acetic acid B: acetonitrile		A: H_2O+0.1% acetic acid B: acetonitrile	
Flow (ml/min)	0.5		0.4		0.4	
Temperature	30		45		45	
Pump-Timetable	time (min)	B (%)	time (min)	B (%)	time (min)	B (%)
	0	33	0	30	0	30
	1	33	0.5	30	0.5	30
	5	80	2	60	2	60
	7	80	2.2	85	2.2	85
	7.5	33	6	85	6	85
	10	33	7	30	7	30
			8	30	8	30
MS-Parameters						
Gas Temp (°C)	350		325		325	
Gas Flow (l/min)	10		10		10	
Nebulizer (psi)	30		20		20	
Sheath Gas Temp. (°C)			350		350	
Sheath Gas Flow (l/min)			11		11	
Capillary (V)	4000		3500		3500	
Nozzle Voltage (V)			1000		1000	
Polarity	negative		negative		negative	
Compounds	Ions	Mass	Ions	Mass	Ions	Mass
	Estrone	269.1	UCA-gluc.	567.3	UCA-gluc.	567.3
	Estrone-gluc.	445.2	DCA-gluc.	567.3	LCA-gluc.	551.3
	Trimegestone-gluc.	517.2	DCA	391.2	LCA	375.2

4.7 Genotyping

4.7.1 Selection of polymorphisms for genotyping

UGT1A3 SNPs for genotyping were selected from a study based on sequencing of a Caucasian population (Caillier et al., 2007). Polymorphisms for *PON*-locus genotyping were derived from dbSNP database (6 coding, 3 promoter, 4 intronic, one 3'UTR SNPs), from the seattle SNP database (6 tagging SNPs with a VAF>0.05 covering 72 SNPs, 1 tagging SNP from this database with VAF>0.02 covering 77 SNPs) and from literature (2 promoter SNPs; Marsillach et al., 2009, 12 intronic SNPs; Saeed et al., 2006, Carlson et al., 2006, Erlich et al., 2006, Campo et al., 2004, Wang et al., 2003, Ranade et al., 2005, Sanghera et al., 2008). Two PON1 coding polymorphisms (L55M, Q192R) were also derived from literature (Dahabreh et al., 2010).

Candidate polymorphisms for genotyping of "Die deutsche Diabetes Dialyse Studie" were selected for a possible influence on the metabolism or transport of atorvastatin from previous

studies and included in a 7-plex assay. To identify carriers of *UGT1A3*2*, rs1983023 (**2* and **6* carriers) and rs45449995 (only present in *6) were selected, as well as, the *PON3* polymorphisms rs11767787 and var55146. Additionally a common upstream polymorphism (*CYP3A4*1B*; -392A>G; rs2740574; VAF=2.5% in Caucasians) was included as one study could show a significantly lowered metabolic activity (Rodríguez-Antona et al., 2005). CYP3A5 is only expressed in approximately 10% of Europeans (Daly, 2006). *CYP3A5**3 (rs776746; VAF=94.2% in Caucasians) is common in all studied populations (rs776746; VAF=94.2% in Caucasians) and explains the absence of CYP3A5 expression in many individuals (Kuehl et al., 2001) and was selected, as well as, *ABCG2* Q141K (rs2231142; VAF=11.7% in Caucasians) resulting in an amino acid change leading to reduced transport activity (Keskitalo et al., 2009). Polymorphisms were assigned by dbSNP rs number, Seattle SNP var number, amino- acid change or naming within literature. Genomic positions refer to reference sequences *ABCG2* (NC_000004.11), *CYP3A4* (NG_008421), *CYP3A5* (NG_007938), *PON3* NG_008726.1 (modified by adding *PON1* NG_008779 and the intergenic region in between), *UGT1A* (AF297093).

4.7.2 MALDI-TOF MS assays

19 *UGT1A3* and 37 *PON*-locus and 7 candidate gene-polymorphisms were genotyped by MALDI TOF MS assays. Primers were designed to specifically amplify *UGT1A3* promoter and exon 1 genomic regions (table 19 genomic position according to AF297093.) or regions around single polymorphisms for the *PON*-locus (table 21**)**, *CYP3A4*, *CYP3A5* and *ABCG2* (table 23) and carried a tag sequence (ACGTTGGATG) to avoid interactions with the MALDI-TOF MS analysis. Correct amplification was confirmed by sequencing. MassArray Assay Design (v3.0.0) was used for the design of MALDI-TOF MS extension primers. Three different assays were developed including 19 *UGT1A3* (table 20), two different assays including 37 *PON*-locus variations (table 22) and one assay including 7 candidate-gene polymorphisms (table 24). Each assay consisted of pre-amplification by PCR, shrimp alkaline phosphatase (SAP) treatment, iPLEX primer extension and a clean resin step. Samples were transferred to a 384 SpectroCHIP® Array (Sequenom) and analyzed in a MassArrayTM Compact mass spectrometer (Sequenom). Automated spectra acquisition was performed using Spectroacquire and data analysis was performed with MassArray Typer software v 3.4.

4.7.3 HPLC-genotyping

Genotyping of the *UGT1A1*28* promoter polymorphism was performed by Christian Klenner applying denaturing HPLC according to a published protocol (Harraway 2005). PCR products

were directly subjected to denaturing HPLC analysis using a DNASepCartridge (column temperature of 64°C) on a WAVE System (Transgenomic, LTD, Crewe, UK). Genotypes were determined from characteristic elution profiles corresponding to one 77 bp fragment (wild-type $(TA)_6$), one 79bp fragment (homozygous $(TA)_7$) or a mixture of both in heterozygous samples. DHPLC results were confirmed by sequencing about 20% of the samples.

4.7.4 Other genotyping

Data for rs757158 (SNP 37) and rs854571 (SNP 38) were obtained from HumanHap300v1.1 chip analysis (Microarray Facility Tübingen Services, Tübingen, Germany). rs705379 (SNP 36) was genotyped using a predesigned TaqMan allelic discrimination assays (C_11708905_10; Applied Biosystems, Darmstadt, Germany) using a TaqMan 7900HT (Applied Biosystems, Darmstadt, Germany).

Table 19: *UGT1A3* amplification primers used for MALDI-TOF MS analysis.

Name	Genomic position	Primer sequence (5'→ 3')	Amplification product (bp)	Purpose
SNP1_f_neu	144852-145219	ACGTTGGATGCCTGGATGACTGAAATAAAG	388	for primer
SNP1_r_neu		ACGTTGGATGCAGCGTGGAGGCTGGCTATG		rev primer
SNP5_f_neu	145477-145927	ACGTTGGATGACTTGGATGTTCCCCAGAGT	471	for primer
SNP5_r_neu		ACGTTGGATGCCTCTGGGGTGAGGACCACT		rev primer
SNP13_f_neu	145934-146495	ACGTTGGATGTGCACATCAAAGAAGAGAAC	582	for primer
SNP13_r_neu		ACGTTGGATGACAGATGCATGACTGAGAAT		rev primer
SNP21_f_neu	146519-146741	ACGTTGGATGTGATGGACTACCCCAGGCCA	243	for primer
SNP21_r_neu		ACGTTGGATGCTGAAGGCTATTATGACAAG		rev primer

Table 20: Extension primers used for MALDI-TOF MS analysis of UGT1A3.

#	Name	Poly-morphism	Genomic position	UGT1A3 Assay	Primer sequence (5'→ 3')	Mass of amplification product (Da)
1	SNP1#1_e_neu	rs55772651	144977	1	CTCCCTGAACCCACC	4417.9
2	SNP1_#2_e_neu	rs1983023	144984	2	CAAGACAACCCTAGCAA	5141.4
3	SNP1#3_e_neu	rs56304713	145154	1	GGATATTTCTTGTAAGGATCA	6475.2
4	SNP1#4_e_W3	rs45507691	145182	3	TGGTTTTGGTCGTTTTT	5219.4
5	SNP5#1_e_neu	rs3806597	145531	1	CCTGGAAAAGACCGATCA	5501.6
6	SNP5#3_e_neu	rs3806596	145669	1	TGCTACATTTGCTTTCTTC	5710.7
7	SNP5#4_e_neu	rs28898617	145751	1	AGTCCTGTGGCCAGCC	4858.2
8	SNP5#5_e_W3	rs3821242	145765	3	CTGAGATGGCCACAGGACTCC	6416.2
9	SNP5#6_e_neu	rs6706232	145815	1	ACCAACACCTTTCCACT	5034.3
10	SNP5_#8_e_neu	rs6431625	145874	2	GCATGGAGCTCCCGCAAG	5509.6
11	SNP13_#1_e_neu	rs17868336	145968	2	ACGAAATGGCATAGGT	4954.3
12	SNP13#2_e_W3	F110I	146062	3	ATTGCCATACTTCTGAAAA	5770.8
13	SNP13#3_e_neu	rs28898619	146076	1	GACATATTGTTCAACATTGC	6091.0
14	SNP13#4_e_W3	A158V	146207	3	CCGTTAACCTCTGCG	4503.9
15	SNP13#5_e_neu	rs7574296	146211	1	TCGACAGGTACTTAGCCAGCAC	6704.4
16	SNP13#6_e_neu	rs45586035	146253	1	GATTCCTACTGTGTTTTTTTT	6374.2
17	SNP13#7_e_neu	D179D	146271	1	AGGAACATTCCATGTGA	5218.4
18	SNP13#8_e_neu	M208L	146356	1	CAACCAATTCAGACCACATGACATTC	7852.1
19	SNP21#_e_neu	rs45449995	146542	1	TACCCCAGGCCAATC	4481.9

Table 21: Amplification primers used in MALDI TOF MS genotyping.

Name	Poly-morphism	Genomic position	Primer sequence (5'→ 3')	Ampli-fication product (bp)
PON3_1f	rs11767787	3882-3982	ACGTTGGATGTAAGCAATCTGTGCTGCAGG	111
PON3_1r			ACGTTGGATGGCTGACACCTATGTTAACGC	
PON3_2f	rs17885453	4148-4256	ACGTTGGATGTCGGTGGAACCTAACAGAAC	119
PON3_2r			ACGTTGGATGACTGAAGATGCGGGAAGA	
PON3_3f	rs17882539	4256-4318	ACGTTGGATGTTCCTCCCCCTCCAACCT	73
PON3_3r			ACGTTGGATGTCCTGCCAGGCAAGAAATG	
PON3_6f	rs2072200	4496-4580	ACGTTGGATGAAGGCAATCGAAGCGAAGAG	95
PON3_6r			ACGTTGGATGAGGTAAGGCACGAAGGTCAG	
PON3_7f	rs17886586	4944-5039	ACGTTGGATGATCCGTACGCGAGGCAGGAA	106
PON3_7r			ACGTTGGATGACGAGCTTCCCCATGGTCTC	
PON3_8f	rs13226149	5058-5156	ACGTTGGATGGGTCGGCCTGTCCTTAGTC	109
PON3_8r			ACGTTGGATGCTCACTTGGAAGAGGAGAG	
PON3_10f	rs10487132	10322-10421	ACGTTGGATGTTCCCACACACTTATTAGCC	110
PON3_10r			ACGTTGGATGACAGGCTAAGAAGCAGTAGG	
PON3_11_12f	rs1053275 rs2375003	29232-29107	ACGTTGGATGAATGAACAAAACCCAAGGGC	136
PON3_11_12r			ACGTTGGATGCCCCTTATCCCTAAACATAC	
PON3_13f	rs468	32673-32769	ACGTTGGATGGTGAGAGTACTTTTCTTCTCC	107
PON3_13r			ACGTTGGATGGTCATCTCCCTTAATTATG	
PON3_14f	rs17879114	33844-33927	ACGTTGGATGGATAGGGGTAACTTTCTTGG	94
PON3_14r			ACGTTGGATGATGTGGGGATGATTCACAAC	
PON3_15f	rs17878827	33928-34038	ACGTTGGATGCCGCACAATACTTTCATTCC	121
PON3_15r			ACGTTGGATGGAAGTCCACTGTGGAGATAT	
PON3_16f	rs9640632	36189-36095	ACGTTGGATGTCAGGCTCCTCTTTAGATCC	105
PON3_16r			ACGTTGGATGCTCTGGGAAGTACATCAGAC	
PON3_17f	rs17883013	37280-37383	ACGTTGGATGGGGAGTTGGTAAAATAGTGG	114
PON3_17r			ACGTTGGATGTGGGTCTCTTTTTCCACCTC	
PON3_18f	rs17880470	37398-37496	ACGTTGGATGGAGATGATCTTGGATCTTCG	109
PON3_18r			ACGTTGGATGTGTGATCCCATTGGCACTAC	
PON3_19f	rs2057682	39847-39951	ACGTTGGATGTTGTACTTTCTCAATGAGGC	115
PON3_19r			ACGTTGGATGAAGGCTCAGCAGAGTAAAGG	
PON3_20f	rs7778771	40313-40426	ACGTTGGATGGATACATAGGATTATTGGAG	124
PON3_20r			ACGTTGGATGAAAGGGTGTCAGTAATTGTG	
PON3_21f	rs17885558	41395-41513	ACGTTGGATGACTCACTGGTTGGTGTTTGC	129
PON3_21r			ACGTTGGATGGAGCTCTAGACTCTAGATAG	
PON3_W2_1f	var1496	1-96	ACGTTGGATGGCAGAAGACATTACTCAGAC	106
PON3_W2_1r			ACGTTGGATGCCTAATCATCATTTTCAGGC	
PON3_W2_3f	var2115	594-688	ACGTTGGATGCAGATTCTCCAAGCCTAGAC	105
PON3_W2_3r			ACGTTGGATGATGTTTAGGTGGAGGGACTG	

PON3_W2_4f	var2375	877-965	ACGTTGGATGTTGAATCTGGAGAGGAAGGC	99
PON3_W2_4r			ACGTTGGATGTGTTACTTCCAGTGGCTTCC	
PON3_W2_9f	var9827	8318-8418	ACGTTGGATGTTGTGCTAGCAGCTGGAAAG	111
PON3_W2_9r			ACGTTGGATGTCCCTTCTCCAACAGAATCC	
PON3_W2_13f	var12788	11306-11405	ACGTTGGATGTGGGCATTCCTGTGGTGTTC	110
PON3_W2_13r			ACGTTGGATGTGGATCCCTATGCTCTCATC	
PON3_W2_14f	rs1003504	11833-11960	ACGTTGGATGGTGTATTTATGAGATGTTG	138
PON3_W2_14r			ACGTTGGATGTTCCAGCAATCAGAATTCAC	
PON3_W2_15f	rs978903	26476-26564	ACGTTGGATGGTATAGAGTGAGAAGGGAGG	99
PON3_W2_15r			ACGTTGGATGCCCAGATAGAAATCCTGCTC	
PON3_W2_16f	Campo219	29001-29088	ACGTTGGATGTGTATATGTGTGCACACTTG	98
PON3_W2_16r			ACGTTGGATGTTTTCCTGGTTCATCTGGCG	
PON3_W2_17f	rs2375002	29283-29404	ACGTTGGATGCCACATAGGGCCAAAAATAC	132
PON3_W2_17r			ACGTTGGATGAACAGGAAGAGAGAAGATGC	
PON3_W2_18f	Wang133	33734-33863	ACGTTGGATGTGGCATTTGTCTGACTTACC	140
PON3_W2_18r			ACGTTGGATGCCAAGAAAGTTACCCCTATC	
PON3_W2_20f	var37120	35612-35710	ACGTTGGATGGAAGGATCCTTCCCTAGAAC	109
PON3_W2_20r			ACGTTGGATGCCAGAAATGTATTGCCTCGC	
PON3_W2_22f	Y233C	38481-38564	ACGTTGGATGTTAGCTGCTACATCAGCTAC	94
PON3_W2_22r			ACGTTGGATGGAGTGTTGTCTCTCATTACC	
PON3_W2_23f	var40512	38982-39093	ACGTTGGATGTTCTTCCAAGTCACCCCAAC	123
PON3_W2_23r			ACGTTGGATGAACTATAACCCTGAGGACCC	
PON3_W2_25f	S311T	41234-41349	ACGTTGGATGTTTCTCGACAGGTACTTCGC	126
PON3_W2_25r			ACGTTGGATGATGGTACACAGAAGCCACAG	
PON3_W2_26f	G324D	41234-41349	ACGTTGGATGTTTCTCGACAGGTACTTCGC	126
PON3_W2_26r			ACGTTGGATGATGGTACACAGAAGCCACAG	
PON3_W2_27f	var45486	(43979-44080)	ACGTTGGATGTTGGATTCCTCCTGGAGTAG	112
PON3_W2_27r			ACGTTGGATGTTGAAGGGAGATGACAAGGC	
PON3_W2_28f	var55146	(53627-53721)	ACGTTGGATGGTGATATTGAAGTCCTCCTC	105
PON3_W2_28r			ACGTTGGATGTCAAAGAACCTAGACCCAGC	
PON3_W2_31f	rs854560	(84581-84698)	ACGTTGGATGTTTCTGGCAGAAACTGGCTC	128
PON3_W2_31r			ACGTTGGATGGCCAGTCCTAGAAAACGTTC	
PON3_W2_32f	rs662	(93191-93291)	ACGTTGGATGGGACCTGAGCACTTTTATGG	111
PON3_W2_32r			ACGTTGGATGTAGACAACATACGACCACGC	

Table 22: Extension primers used in *PON*-locus MALDI-TOF MS genotyping.

#	Name	Poly-morphism	Genomic position	*PON*-locus assay	Primer sequence (5'→ 3')	Mass of ampli-fication product (Da)
1	PON3_W2_1e	var1496	42	2	ggCCTTTCTTAAGAAAGGGCTAAT	7391.8
2	PON3_W2_3e	var2115	661	2	CACCACCCCTTTGCTCATATCCAA	7152.7
3	PON3_W2_4e	var2375	921	2	GTAGGCCAAGTTAAGAAAC	5869.9
4	PON3_W2_5e	rs11767787	3935	2	AATTATCAACACAATCTCTGGAG	7015.6
5	PON3_2e	rs17885453	4232	1	TGCTACTTTGCCCGAACT	5425.5
6	PON3_3e	rs17882539	4280	1	CCTCCAACCTGGTGTT	4808.1
7	PON3_6e	rs2072200	4528	1	ATCTTCTCCAGGATTTGGGGCAC	7030.6
8	PON3_8e	rs13226149	5088	1	agTAGTCGGGGAGATGTT	5634.7
9	PON3_W2_9e	var9827	8372	2	ACGCCTTTCCTGAATT	4807.1
10	PON3_10e	rs10487132	10383	1	cGCCTATGCACAACTATCATTA	6638.3
11	PON3_W2_13e	var12788	11333	2	GGCTTTTTTAGTTGACTGGTTTACCC	7949.2
12	PON3_W2_14e	rs1003504	11895	2	ggATTTGTTAAATCAATTGCATTTTG	8005.2
13	PON3_W2_15e	rs978903	26521	2	TCGATAAAAACAGAAGGAGG	6232.1
14	PON3_W2_16e	Campo219	29055	2	AAAAGGGATTAAAATATCCAGG	6824.5
15	PON3_11e	rs1053275	29133	1	ggAACCCAAGGGCACAAGC	5840.8
16	PON3_12e	rs2375003	29155	1	CCATGTGGATTAAATAATTCTTTGT	7661
17	PON3_W2_17e	rs2375002	29330	2	cCAAGGTTTTATACCTATTTATCATTT	8189.4
18	PON3_13e	rs468	32735	1	gTTCTCCATCTCCTCATTCC	5929.9
19	PON3_W2_18#1e	Wang133	33772	2	ACCTATCATGTAGACTGTGAG	6445.2
20	PON3_W2_18#2e	rs3757708	33797	2	TTTCTTCTTACATCTTGCATTT	6607.3
21	PON3_14e	rs17879114	33898	1	gTTCCATGTAGACAATACTGT	6420.2
22	PON3_15e	rs17878827	33956	1	AGAGAACGTTGTTGTTCCT	5833.8
23	PON3_W2_20e	var37120	35664	2	CATGGCCCTACCAATAACAC	6014.9
24	PON3_16e	Campo219	36134	1	GTTCCAGCTGCTGCTA	4848.2
25	PON3_17e	rs2375002	37354	1	AATAGTGGTCTCTGGTG	5256.4
26	PON3_18e	rs17880470	37427	1	cGATCTTCGCTGGACTTA	5465.6
27	PON3_W2_22e	Y233C	38537	2	GCTGCTACATCAGCTACATAGACA	7305.8
28	PON3_W2_23e	var40512	39056	2	ACCCCAACAAATTTGTTC	5402.5
29	PON3_19e	rs2057682	39924	1	TTCTCAATGAGGCCTACTCT	6042.9
30	PON3_20e	rs7778771	40352	1	GGAGAATTGTTTAGGATCTTTTT	7114.6
31	PON3_W2_25e	S311T	41269	2	CGCATCCAGAATGTTTTG	5489.6
32	PON3_W2_26e	G324D	41309	2	ttCACCGTGTATGCCAACAATG	6694.4
33	PON3_21e	rs17885558	41438	1	CAATTATCAGTTTACTTTTACAAAATAT	8519.6
34	PON3_W2_27e	var45486	44028	2	CCATTCTTTCCAAAGGATAG	6076
35	PON3_W2_28e	var55146	53688	2	gACCTTGAGGCAATGTG	5250.4
39	PON3_W2_31e	rs854560	84608	2	AACTGGCTCTGAAGAC	4890.2
40	PON3_W2_32e	rs662	93246	2	gTTCTTGACCCCTACTTAC	5689.7

Table 23: Amplification primers used for MALDI-TOF MS analysis of samples of the "4-D"-study.

Name	Poly-morphisms	Genomic position	Primer sequence (5'→ 3')	Ampli-fication product (bp)
SNP1_A_f_neu	rs1983023	144852-145219	ACGTTGGATGCCTGGATGACTGAAATAAAG	367
SNP1_r_neu			ACGTTGGATGCAGCGTGGAGGCTGGCTATG	
SNP21_f_neu	rs45449995	146519-146741	ACGTTGGATGTGATGGACTACCCCAGGCCA	222
SNP21_r_neu			ACGTTGGATGCTGAAGGCTATTATGACAAG	
ABCG2_f	rs2231142	27636-27804	ACGTTGGATGCATTACCTTGGAGTCTGCCA	169
ABCG2_r			ACGTTGGATGTTGCCTTAAGGATGATGTTG	
CYP3A4*1B_f	rs2740574	4640-4839	ACGTTGGATGGAGTTCATATTCTATGAGGT	200
CYP3A4*1B_r			ACGTTGGATGAGATCTGTAGGTGTGGCTTG	
CYP3A5*3_f	rs776746	11909-12186	ACGTTGGATGGGTTCTAGTTCATTAGGGTG	277
CYP3A5*3_r			ACGTTGGATGCCTGCCTTCAATTTTTCACT	
PON3_1f	rs11767787	3882-3982	ACGTTGGATGTAAGCAATCTGTGCTGCAGG	100
PON3_1r			ACGTTGGATGGCTGACACCTATGTTAACGC	
PON3_W2_28f	var55146	(53627-53721)	ACGTTGGATGGTGATATTGAAGTCCTCCTC	94
PON3_28r			ACGTTGGATGCCAGTCAAACATGCTGTAGG	

Table 24: Extension primers used for MALDI-TOF MS analysis of samples of the "4D"-study.

Name	Poly-morphisms	Genomic position	4D- assay	Primer sequence (5'→ 3')	Mass of amplification product (Da)
SNP21#_e_neu	rs1983023	146542	1	TACCCCAGGCCAATC	4481.9
SNP1#2_e_neu	rs45449995	144984	1	CAAGACAACCCTAGCAA	5141.4
PON3+12285_e	var55146	(53688)	1	CAGCAGCACAATGGTGG	5244.4
PON3-1091_e	rs11767787	3935	1	cTCAACACAATCTCTGGAG	5756.8
ABCG2_e	rs2231142	27688	1	CGAAGAGCTGCTGAGAACT	5861.8
CYP3A5*3_e	rs776746	12083	1	GGTCCAAACAGGGAAGAGATA	6537.3
CYP3A4*1B_e	rs2740574	4713	1	gtACAGCCATAGAGACAAGGGCA	7115.7

4.8 Atorvastatin pharmacokinetics *in vivo*

The pharmacokinetics of atorvastatin and its metabolites were characterized by the peak concentration in plasma (C_{max}), time to C_{max} (t_{max}), elimination half-life ($t_{½}$), and area under the plasma concentration-time curve from 0 h to infinity ($AUC_{0-\infty}$) originated from published studies of a collaborator (Keskitalo et al., 2008; Keskitalo et al., 2009). In short, after an

overnight fast, 56 healthy volunteers received a single 20 mg dose of atorvastatin (Lipitor, Pfizer/Gödecke, Karlsruhe, Germany) with 150 ml water at 8 AM. The use of other drugs was prohibited for one week and in addition the use of grapefruit products for three days before atorvastatin administration. Blood samples (5 to 10ml each) were drawn at specified time points prior to and for up to 48h after atorvastatin ingestion into tubes that contained EDTA. Samples were transferred on ice immediately after sampling. Plasma was separated within 30 minutes after blood sampling and stored at -70°C until analysis. Plasma concentrations of atorvastatin and its metabolites were determined on a SCIEX Q Trap LC-MS-MS system (Sciex Division of MDS, Toronto, Ontario, Canada), as described in these studies (Keskitalo et al., 2008; Keskitalo et al., 2009). All studies involving humans (including liver donors) were approved by the "Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa, Finland", and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from the participants.

4.9 Statistical analysis and computational genetic analysis

GraphPad Prism v4.00 (GraphPad Software Inc., San Diego, CA) was applied for Spearman correlation analysis of activity data and expression phenotypes. Effects of non-genetic factors on enzymatic activity and mRNA/protein expression were tested by Wilcoxon-Mann-Whitney-Tests. Fold-changes are representing observed differences between mean values of corresponding phenotypes.

Observed and expected allele and genotype frequencies within populations were tested for deviation from Hardy-Weinberg equilibrium using the online DeFinetti program (Tim M. Strom and Thomas F. Wienker, 2009). Linkage disequilibrium (LD) plots were created by Haploview 4.2 “Haploview | Broad Institute of MIT and Harvard,” 2009. Haplotype computations of *UGT1A3* were performed by PHASE version v2.1.1. For *UGT* allele nomenclature this work followed published recommendations (“Nomenclature: UDP Glucuronosyltransferase Alleles,” 2011). Haplotypes for the *PON*-locus were generated with the help of Stefan Winter using statistical software R v2.11.1 (“The R Project for Statistical Computing,” 2011) with the library haplo.stats v1.4.4. Exclusion criterion for polymorphisms was a VAF<2%. In case of 100% linked polymorphisms, only one representative was chosen and, if missing data occurred, completed by the 100% correlated polymorphisms. An iterative two-step expectation-maximization algorithm was applied to predict haplotype assignment of individuals based on activity data and polymorphisms (log-additive genetic model). Rare haplotypes were defined by a haplotype frequency<2% and were not included in further analyses. Resulting 13 haplotypes were manually combined to seven major types according to

occurrence of *PON1* promoter and *PON3* F21 (rs13226149) and four promoter polymorphism. Table 10, section 2.3.8 shows the final haplo- and sub-types and their predicted frequencies in the Caucasian population. Wilcoxon-Mann-Whitney-Tests were used to compare a) carriers of identified haplotypes vs. carriers of reference haplotypes (**1* or **1/*1*) and b) for each of the selected *PON1/3* polymorphisms, heterozyote and homozygote carriers of variant allele vs. homozygote carriers of reference allele.

Moreover, multivariate linear models and step-wise model selection based on Akaike's information criterion were applied to determine the contribution of polymorphisms and non-genetic factors to activity and expression variability. In the PON analysis, atorvastatin-lactone hydrolysis and PON1 and PON3 expression were first log-transformed in order to satisfy Gaussian distribution assumption. Normality of log-transformed values was verified by normal quantile-quantile plots. Moreover, for each polymorphism, the genetic model (dominant, recessive or log-additive) with the most significant effect on the corresponding phenotype in the univariate analyses was chosen. Relevant polymorphisms in the PON analysis were: *PON3 -4984* (var1496), *-1091* (rs11767787), F21 (rs13226149), *PON1 -1741* (rs757158) and *-108* (rs705379). The coefficients of determination adjusted for the number of factors in the considered models were then used to specify the fraction of phenotype variability explained by a) only polymorphisms, b) only non-genetic factors and c) both, polymorphisms and non-genetic factors.

All tests were two-sided and statistical significance was defined as $P<0.05$.

Atorvastatin pharmacokinetic data were expressed as estimated marginal mean values adjusting for covariates with 95% confidence intervals (CI), or as median with range (t_{max}), and, for clarity, as estimated marginal mean (± SEM; figure 28). Logarithmically transformed C_{max}, $t_{½}$, and $AUC_{0-\infty}$ values were compared between different *UGT1A3* genotypes using ANOVA with body weight, sex, and the *ABCB1* c.1236C>T, c.2677G>T/A, and c.3435C>T, *SLCO1B1* c.521T>C, and *ABCG2* c.421C>A SNPs as covariates (Pasanen et al., 2007; Keskitalo et al., 2008; Keskitalo et al., 2009). *A priori* pairwise comparisons between the *UGT1A3* genotypes were made using the Fisher's least significant difference method. The t_{max} data were analyzed with the Kruskal-Wallis test with *a priori* pairwise testing with the Mann-Whitney test. All tests were two-sided and differences were considered statistically significant when *P* was below 0.05. Statistical significance was assigned by * when compared with *UGT1A3*1/*1* genotype, by † when compared with *UGT1A3*1/*2* genotype, by ‡ when compared with *UGT1A3*2/*2* genotype

and by § when compared with *UGT1A3*1/*3* genotype.

The occurrence of primary study endpoints was analyzed by Kaplan-Meier plots and significance of genotype differences was tested using log-rank tests. Hazard ratios were determined with cox regression analyses.

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6. Publications:

6.1 Publications in peer reviewed journals

6.1.1 Distinction between human cytochrome P450 (CYP) isoforms and identification of new phosphorylation sites by mass spectrometry.

Redlich G, Zanger UM, Riedmaier S, Bache N, Giessing AB, Eisenacher M, Stephan C, Meyer HE, Jensen ON, Marcus K.
J Proteome Res. 2008 Nov;7(11):4678-88.

6.1.2 UDP-glucuronosyltransferase (UGT) polymorphisms affect atorvastatin lactonization in vitro and in vivo.

Riedmaier S, Klein K, Hofmann U, Keskitalo JE, Neuvonen PJ, Schwab M, Niemi M, Zanger UM.
Clin Pharmacol Ther. 2010 Jan; 87(1):65-73.

6.1.3 Profiling induction of cytochrome p450 enzyme activity by statins using a new liquid chromatography-tandem mass spectrometry cocktail assay in human hepatocytes.

Feidt DM, Klein K, Hofmann U, Riedmaier S, Knobeloch D, Thasler WE, Weiss TS, Schwab M, Zanger UM.
Drug Metab Dispos. 2010 Sep; 38(9):1589-97.

6.1.4 Systems Biology Approach to Dynamic Modeling and Inter-Subject Variability of Statin Pharmacokinetics in Human Hepatocytes.

Joachim Bucher, Stephan Riedmaier, Anke Schnabel, Katrin Marcus, Andreas Nüssler, Ulrich M Zanger, Matthias Reuss
BMC Syst Biol. 2011 May; 5(1):66.

6.1.5 Paraoxonase (*PON1* and *PON3*) polymorphisms: impact on liver expression and atorvastatin-lactone hydrolysis

S Riedmaier, K Klein, S Winter, U Hofmann, M Schwab, UM Zanger
Frontiers in Pharmacogenetics and Pharmacogenomics.

6.2 Posters and Talk

6.2.1 Conference on Systems Biology of Mammalian Cells, Dresden, May 2008

"Investigation of Hepatic Metabolism of the HMGCoA Reductase Inhibitor Atorvastatin"
Stephan Riedmaier, Jessica Rieger, Kathrin Klein, Tanja Saussele, Ulrich M. Zanger

6.2.2 11th European Regional ISSX Meeting, Lisbon, Portugal, May 2009

"Atorvastatin Lactonization Catalyzed by UGT1A3:
Influence of Genotype and Implications for Drug Response"
Stephan Riedmaier, Mikko Niemi, Ute Hofmann, Kathrin Klein, Ulrich M. Zanger

6.2.3 HepatoSys – Konferenz des Netzwerkes Systembiologie, Berlin, January 2009

"Quantitative, Genetic and Population Approaches for
Systems Biology of Statins in Human Liver"
Stephan Riedmaier, Kathrin Klein, Ulrich M. Zanger

6.2.4 logP 2009 – PhysChem and ADMET Profiling in Drug Research, Zürich, January 2009

"Metabolism and Transport of the HMGCoA Reductase Inhibitor Atorvastatin"
Stephan Riedmaier, Matthias Schwab, Ulrich M. Zanger

6.2.5 POSTER-PRIZE: 11. Jahreskongress für klinische Pharmakologie, Heidelberg, October 2009

"Influence of UDP-Glucuronosyltransferase (UGT)-Polymorphisms on Atorvastatin
Lactonization and Implications for Drug Response"
Stephan Riedmaier, Kathrin Klein, Ute Hofmann, Jenni E. Keskitalo, Pertti J. Neuvonen, Matthias Schwab, Mikko Niemi, Ulrich M. Zanger

6.2.6 Talk: 11. Jahreskongress für klinische Pharmakologie, Heidelberg, October 2009

"UDP-Glukuronosyltransferase 1A3 Polymorphismen:
Ein neuer Prädiktor für Wirkung und Toxizität von Statinen?"
Stephan Riedmaier

6.2.7 Microsomes and Drug Oxidations, Peking, May 2010

"Novel Potential Determinants of Atorvastatin Efficacy and Toxicity:
Variable Formation and Hydrolysis of Acyl-Lactone via UGT1A3 and PON3"
Stephan Riedmaier, Kathrin Klein, Ute Hofmann, Matthias Schwab, Ulrich M. Zanger

6.3 Patent

Pub. No.: WO/2010/108737
International Application No.: PCT/EP2010/052245
Publication Date: 30.09.2010
International Filing Date: 23.02.2010

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(DE) VERFAHREN ZUR BESTIMMUNG DER PRÄDISPOSITION EINES PATIENTEN ZU VERÄNDERTER BIOTRANSFORMATION UND ZUR ENTWICKLUNG VON UNERWÜNSCHTEN ARZNEIMITTELWIRKUNGEN BEI EINER BEHANDLUNG DES PATIENTEN MIT ATORVASTATIN

(EN) METHOD FOR DETERMINING THE PREDISPOSITION OF A PATIENT TO CHANGED BIOTRANSFORMATION AND TO THE DEVELOPMENT OF UNDESIRED PHARMACEUTICAL EFFECTS IN A TREATMENT OF THE PATIENT WITH ATORVASTATIN

(FR) PROCÉDÉ DE DÉTERMINATION DE LA PRÉDISPOSITION D'UN PATIENT À UNE BIOTRANSFORMATION MODIFIÉE ET AU DÉVELOPPEMENT D'EFFETS SECONDAIRES INDÉSIRABLES LORS D'UN TRAITEMENT PAR ATORVASTATIN

Abstract:

(DE) Die vorliegende Erfindung betrifft ein Verfahren zur Bestimmung einer Prädisposition eines Patienten für die Entwicklung von Muskelerkrankungen und/oder zu veränderter Biotransformation bei einer Behandlung des Patienten mit Atorvastatin. Dabei wird in einer biologischen Probe des Patienten das Vorliegen von zumindest einem Einzelnucleotid-Polymorphismus (SNP) im UGT1A3-Gen (Uridindiphosphat- Glucuronosyltransferase-Gen 1A3) und/oder eine erhöhte UGT1A3-Genexpression bestimmt. Ferner betrifft die Erfindung Oligonucleotide, die bei dem Verfahren eingesetzt werden können, sowie diagnostische Kits, die diese Oligonucleotide verwenden.

(EN) The present invention relates to a method for determining a predisposition of a patient to the development of muscular diseases and/or to changed biotransformation in a treatment of

the patient with atorvastatin. The presence of at least one single nucleotide polymorphism (SNP) in the UGT1A3 gene (uridine-diphosphate-glucuronosyltransferase gene 1A3) and/or an increased UGT1A3 gene expression is determined in a biological sample of the patient. The invention further relates to oligonucleotides that can be used in the method and to diagnostic kits that use said oligonucleotides.

(FR) La présente invention concerne un procédé de détermination de la prédisposition d'un patient à développer des myopathies et/ou de sa prédisposition à une biotransformation modifiée lors d'un traitement du par Atorvastatin. On détermine dans un échantillon biologique du patient l'existence d'au moins un polymorphisme nucléotidique simple (SNP) dans le gène UGT1A3 (gène de uridindiphosphate- glucuronosyltransférase 1A3) et/ou l'existence d'une expression accrue du gène UGT1A3. En outre, l'invention concerne des oligonucléotides qui peuvent être utilisés dans ce procédé, ainsi que des kits de diagnostic qui utilisent ces oligonucléotides.

7. CURRICULUM VITAE of Stephan Riedmaier

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PROFESSIONAL EDUCATION

01/2008 – 08/2011	doctoral thesis entitled: "**Pharmacogenetic Determinants of Atorvastatin Metabolism and Response"** Dr. Margarete Fischer-Bosch institute of clinical pharmacology, Stuttgart, Germany
10/2001 – 09/2007	study of "technische Biologie" at the University of Stuttgart major subject: experimental biology physiology of animals molecular biology minor subjects: biochemistry industrial genetics bioprocess engineering diploma thesis entitled: **„Membranvesikel aus Lebergewebe und Hepatozyten in Sandwich-Kultur: Etablierung experimenteller Systeme zur Untersuchung des hepatischen Transportes von Pharmaka"** Dr. Margarete Fischer-Bosch institute of clinical pharmacology, Stuttgart, Germany degree: diploma biologist, technically oriented

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COLLEGE EDUCATION

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8. Acknowledgment – Danksagung:

Als erstes möchte ich Professor Ulrich Zanger für die Möglichkeit danken, an diesem interessanten Thema zu arbeiten, sowie für die wertvollen Diskussionen im Verlauf der Promotion, seine lektorische Unterstützung, sowie persönlichen, wie wissenschaftlichen Rat.

Dank gilt auch Professor Matthias Schwab, dem Leiter des Dr. Margarete Fischer-Bosch Instituts für klinische Pharmakologie für die Möglichkeit, meine Dissertation an diesem Institut machen zu können, aber auch für viele hilfreiche Anregungen.

Professor Michael Schwarz danke ich herzlich für seine Bereitschaft, die Arbeit zu Betreuen und zu Begutachten.

Danken möchte ich auch Dr. Kathrin Klein für die vielen fundierten Ratschläge, wann immer sie nötig waren. Ebenso danke ich den Kollegen aus der Analytik, Dr. Ute Hofmann, Dr. Thomas Mürdter, Monika Seiler und Sonja Seefried, für ihre Hilfe beim Aufbau analytischer Methoden. Stefan Winter danke ich für seine Hilfe die Statistik betreffend, und die Organisation unserer Badminton-Spiele. Meinen Kollegen Britta Klumpp und Igor Liebermann danke ich für ihre Unterstützung und für ihre Hilfe beim erlernen praktischer Methoden. Auch danke ich meiner ehemaligen Kollegin Dr. Diana Feidt für die gemeinsamen, meist sehr konstruktiven Kaffeepausen.

Danke auch an meine Büro-Kollegen Barbara van Eijck, Dr. Susanne Karner, Stephanie Kopacz, Jessica Rieger und Claudia Resch, sowie an die tägliche Mittagessens-Runde für die schöne Zeit und die interessanten Gespräche. Außerdem möchte ich mich bei der gesamten AG Zanger und dem IKP Team für die nette Arbeitsatmosphäre und ständige Hilfsbereitschaft danken.

Ich danke Arian für ihre Hilfe und dafür, dass Sie mich motivierte, wann immer die Motivation fehlte. Auf die Zukunft mit Dir freue ich mich sehr.

Zum Schluss danke ich meinen Eltern und meiner Familie für ihre Unterstützung während meiner Schulzeit, meines Studiums und der Promotion. Eure Hilfe bot mir immer großen Rückhalt.

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Stuttgart, 09.06.2011

Stephan Riedmaier