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Expression profile and pharmacogenetics of genes candidate to play a role in cardiovascular disease

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Tese orientada pela Professora Doutora Vera Linda Ribeiro Marques

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Contents

Abstract

Cardiovascular diseases are the major causes of death and morbidity in developed countries. Several risk factors have been associated to cardiovascular diseases, but the molecular mechanisms underlying the alteration of homeostasis are still largely unknown. There is increasing recent evidence that the drug metabolizing enzyme system associated to a number of membrane transport proteins is of particular relevance in relation to cardiovascular diseases, either in biosynthetic or degradative pathways.

The overall aim of the present study was to characterize selected genes coding for drug metabolizing enzymes and influx and efflux transporters both from the point of view of pharmacogenetic diversity and from their response to increased cholesterol concentrations. Specifically we a) characterized, in a sample of the Portuguese population, selected genetic polymorphisms in cytochromes P450, influx and efflux transporters that are known to – or may be expected to - affect *in vitro* or *in vivo* the metabolism or transport of cholesterol and/or therapeutic drugs; b) evaluated the existence of ethnic variability in the frequency of these polymorphic variants, through the determination of their prevalence in other populations; and c) investigated to which extent the expression of these genes, in liver and intestine derived cells, may be influenced by the accumulation of cholesterol.

Most of the polymorphisms analysed present a similar distribution between Portuguese and Colombian populations, but significant differences when compared with the Mozambican population, demonstrating that these polymorphisms are ethnicity-dependent.

The expression profiling studies revealed a differential expression pattern for the analysed genes in cell culture models from liver and intestine and a differential response to cholesterol, pointing to significant differences in gene regulation in these tissues.

Taken together, the results obtained in this study contribute to a better knowledge of drug metabolism and transport in hypercholesterolemia as well as to understanding the pharmacogenetic variability of the genes involved in these pathways.

Keywords:

Cytochrome P450 (CYP)

Influx transporters (SLC)

Efflux transporters (ABC)

Cholesterol

Pharmacogenetics

Resumo

As doenças cardiovasculares são a maior causa de morte e morbilidade nos países desenvolvidos. Embora vários factores de risco estejam associados às doenças cardiovasculares, como pressão sanguínea elevada, obesidade, níveis de colesterol elevados, diabetes, stress e tabaco, os mecanismos moleculares subjacentes às alterações da homeostasia envolvidas não são ainda conhecidos. A prevenção das doenças cardiovasculares baseia-se na terapêutica de redução de lípidos, estando diversos fármacos disponíveis para este propósito, como as estatinas, inibidores da absorção de colesterol, resinas de ácidos biliares, fibratos e ácido nicotínico, os quais actuam no organismo em diferentes vias, tendo por isso diferentes modos de acção entre si.

Um número crescente de publicações tem vindo a demonstrar que o sistema de enzimas metabolizadores de xenobióticos (citocromos P450), associado a proteínas de transporte membranar, como a superfamília de transportadores dependentes de ATP (transportadores ABC) ou a superfamília de transportadores de influxo (SLC), é de particular relevância em relação às doenças cardiovasculares, tanto em termos de vias de biossíntese, como das vias de catabolismo.

O fígado, sendo o principal órgão de destoxificação do organismo, extrai os xenobióticos do sangue através de transportadores membranares especializados, os transportadores de influxo, tornando estes compostos disponíveis para biotransformação pelos enzimas metabolizadores. Assim, os citocromos P450 modificam o substrato, geralmente aumentando a sua solubilidade, facilitando desta forma, a sua eliminação. Os produtos metabólicos são então excretados da célula através dos transportadores de efluxo ABC.

Os transportadores de influxo (SLC) são uma superfamília de transportadores que facilita a passagem de solutos ao longo das membranas celulares, encontrando-se presentes em diversos órgãos. Alguns transportadores de influxo apresentam uma expressão elevada no fígado, órgão no qual desempenham um papel crucial na disposição e eficácia de muitos fármacos, como as estatinas, usadas no tratamento de doenças cardiovasculares. Além de transportar xenobióticos, os transportadores de influxo desempenham papeis fisiológicos fundamentais, através do transporte de compostos endógenos importantes.

Os citocromos P450 são um grupo de proteínas membranares que estão envolvidos no metabolismo de diversos compostos endógenos e exógeno, dando origem a produtos que desencadeiam respostas fisiológicas.

Níveis elevados de colesterol são um factor de risco bem estabelecido para as doenças cardiovasculares e, estes isoenzimas podem ainda ter um papel importante no risco destas doenças, uma vez que participam nos mecanismos que controlam a homeostasia do colesterol, catalisando passos chave no metabolismo deste composto, tanto na sua síntese como na sua eliminação através da sua conversão a metabolitos, como os ácidos biliares. Dois exemplos do papel destes enzimas, são a conversão de lanosterol a colesterol catalisada pelo CYP51 e o

passo limitante para a conversão do colesterol a ácidos biliares, hidroxilação do colesterol na posição 7α, catalisada pelo CYP7A1.

Para além da sua função na homeostasia de compostos endógenos, os citocromos P450 podem ser de crucial importância no metabolismo de fármacos utilizados no tratamento de doenças cardiovasculares. A variabilidade na inactivação metabólica pode ser bastante importante na falha terapêutica, uma vez que os fármacos mais eficientes no tratamento das doenças cardiovasculares são metabolizados pelos citocromos P450.

Os transportadores ABC são transportadores activos, que dependem da hidrólise de ATP para transportar um substrato ao longo da membrana plasmática. Os transportadores ABC têm sido associados ao transporte de ácidos biliares, fosfolípidos e esteróis, onde a própria expressão dos transportadores é controlada pelos níveis lipídicos.

Deste modo, e porque os genes destes transportadores de efluxo estão associados a diversas doenças genéticas em humanos, os transportadores ABC são candidatos promissores para o tratamento de desordens lipídicas, como as doenças cardiovasculares.

Estes enzimas metabolizadores de xenobióticos, juntamente com os transportadores de influxo e de efluxo são membros de famílias multigénicas, nas quais os seus genes codificam proteínas que possuem uma grande diversidade de substratos. No seu conjunto, este sistema metabólico compreende um grande número de diferentes componentes e, devido à sua relação metabólica, surge a possibilidade da sua regulação ser cruzada (cross talk regulation), relacionada em particular ao nível da transcrição de genes induzida por receptores nucleares. Os receptores nucleares, tais como o LXR, FXR, PXR, CAR, ROR, HNF4 e PPAR parecem ter um papel importante na regulação transcripcional de vários transportadores de influxo e de efluxo, assim como dos enzimas metabolizadores de xenobióticos. Estes receptores são activados por uma grande diversidade de ligandos, tais como ácidos biliares e oxiesteróis (metabolitos do colesterol), sendo reguladores chave no metabolismo dos lípidos. Para além dos receptores nucleares existem outras moléculas que também podem regular o metabolismo lipídico, como o SREBP, um factor de transcrição envolvido na regulação de genes envolvidos na biosíntese e metabolismo do colesterol.

Os genes que codificam estes enzimas metabolizadores de xenobióticos e transportadores de influxo e de efluxo são polimórficos, levantando a possibilidade de que estas variações genéticas inter-individuais actuam como factores de susceptibilidade para as doenças cardiovasculares, assim como para falhas terapêuticas ou efeitos adversos associados a fármacos. O conhecimento dos marcadores farmacogenéticos para estas doenças, juntamente com os mecanismos de regulação dos genes envolvidos, pode contribuir para uma melhor vigilância e para o desenvolvimento de estratégias de prevenção, assim como para a identificação de potenciais reguladores úteis no desenvolvimento de novas estratégias terapêuticas.

O objectivo geral do presente estudo foi caracterizar um conjunto de genes que codificam enzimas metabolizadores e transportadores de influxo e de efluxo, tanto do ponto de vista da diversidade farmacogenética como da sua resposta a um aumento na concentração de colesterol. Especificamente nós a) caracterizámos, numa amostra da população Portuguesa, polimorfismos genéticos em citocromos P450, transportadores de influxo e de efluxo, que comprovadamente ou potencialmente afectem *in vitro* ou *in vivo* o metabolismo ou o transporte de colesterol e/ou fármacos; b) avaliámos a existência de variabilidade étnica na frequência destas variantes polimórficas, através da determinação da sua prevalência noutras populações; e c) investigámos a possível existência de variações na expressão desses genes, em células derivadas do fígado e do intestino, numa situação de aumento na concentração de colesterol.

Os componentes do sistema de destoxificação apresentam um elevado grau de variabilidade inter-individual, a qual determina os níveis circulantes dos substratos correspondentes. Isto reflecte a existência de uma diversidade estrutural nos genes correspondentes – polimorfismos genéticos – que podem determinar o risco individual para uma dada patologia e/ou a eficácia da estratégia terapêutica. Neste estudo foram desenvolvidos métodos específicos para identificar variantes relevantes nos genes que codificam para os transportadores de influxo, efluxo e para os enzimas metabolizadores de xenobióticos e caracterizámos a sua prevalência na população Portuguesa. De seguida, comparámos a prevalência desses polimorfismos na população Portuguesa com indivíduos de locais geográficos distintos, de Moçambique e da Colômbia.

A genotipagem foi realizada por PCR-RFLP (*Polymerase chain reaction-restriction fragment length polymorphism*) para os enzimas (*CYP7A1-* A-203C, C-278A, C-496T, A698G, G1039A; *CYP7B1-* C-116G, C-1774T, G971A; *CYP27A1-* C490T, C506T, G817A, T1633C), e para os transportadores de influxo (*SLCO1B1-* T217C, A388G, C463A, T521C; *SLCO1B3-* T334G, G699A, G1564T; *SLC22A1-* C480G, C886G, T4215C; *SLC10A1-* C800T, A2587G, A2192G) e efluxo (*ABCB11-* T580C, G779A, C1331T, A2155G; *ABCG5-* G80C, C148T, C1550G, C1810G; *ABCG8-* G55C, A161G, C1199A, T1895C).

Não foram identificados indivíduos portadores do alelo variante para os polimorfismos *CYP7A1* A698G, *CYP7B1* C-1774T, *SLC10A1* C800T, *SLCO1B1* T217C e *ABCB11* G677A, em qualquer das três populações analisadas, o que pode indicar que estes polimorfismos possam estar localizados em regiões conservadas que desempenham um papel funcional essencial na proteína.

A maioria dos polimorfismos apresentou uma distribuição semelhante entre as populações de Portugal e Colômbia, sendo no entanto observadas diferenças significativas quando se compara estas populações com a população de Moçambique. Estas diferenças demonstram que a prevalência destes polimorfismos é dependente da etnia, sugerindo que as diferenças nas frequências alélicas podem ser uma consequência da selecção natural nalgumas localizações geográficas, o que pode ter tido um papel na diversidade em alguns loci.

De salientar ainda que alguns dos polimorfismos analisados neste estudo, representam a sua primeira descrição na literatura, nomeadamente *SLC22A1* C886G e T4215C; *SLC10A1* A2587G e A2192G; *CYP7A1* T-346C, A698G e G1039A; *CYP7B1* G971A e C-1774T; *CYP27A1* C490T, C506T, G817A e T1633C; *ABCG5* G80C, C148T e C1550G.

Os resultados obtidos neste estudo contribuem para uma melhor compreensão dos perfis genéticos das três populações analisadas, Portugal, Moçambique e Colômbia. De facto, alguns dos polimorfismos analisados foram previamente descritos como sendo clinicamente importantes, podendo contribuir para a variabilidade no tratamento, realçando a importância da sua caracterização em diferentes populações.

Os estudos farmacogenéticos são de extrema importância, uma vez que abrem a porta para um novo tipo de tratamento, a terapêutica personalizada. O conhecimento do perfil genético de uma determinada população permite o desenvolvimento de uma nova terapia, mais segura e eficaz para cada indivíduo. Deste modo, a caracterização das frequências alélicas de determinados polimorfismos em diferentes etnias, é útil para prever as alterações na farmacocinética de fármacos e, em consequência, as diferentes susceptibilidades a possíveis efeitos adversos.

O colesterol é uma molécula essencial para o controlo da fluidez das membranas celulares, como substrato para a síntese de ácidos biliares e hormonas esteróis, desempenhando deste modo, um papel crucial nas células dos mamíferos. No entanto, as necessidades celulares assim como a ingestão de colesterol são muito variáveis, e por isso a os processos envolvidos na manutenção da sua homeostasia são altamente regulados. Com vista a avaliar a resposta destes genes ao colesterol, procedeu-se à determinação dos correspondentes padrões de expressão em modelos celulares de origem hepática ou intestinal, nomeadamente células HepG2 (hepatoma) e Caco-2 (adenocarcinoma do cólon). As condições de cultura e os protocolos de sincronização foram optimizados de forma a adaptar as células a um meio sem soro para minimizar variáveis e optimizar os resultados, uma vez que alguns compostos, como o colesterol, que estão presentes no soro, podiam interferir com os resultados. As células foram posteriormente tratadas com concentrações de colesterol diferentes, durante vários períodos de tempo, sendo então feita a extracção do mRNA. Os níveis de mRNA dos enzimas metabolizadores (*CYP3A4, CYP7A1, CYP27A1, CYP39A1, CYP51A1, CYP7B1, CYP8B1*), e dos transportadores de influxo (*SLC10A1* (NTCP), *SLC22A1* (OCT-1), *SLC22A7* (OAT2), *SLCO1A2* (OATP-A), *SLCO1B1* (OATP-C), *SLCO1B3* (OATP-8), *SLCO2B1* (OATP-B)) e efluxo (*ABCA1, ABCB11* (BSEP), *ABCC2* (MRP2), *ABCC3* (MRP3), *ABCC6* (MRP6), *ABCG2* (BCRP), *ABCG5, ABCG8*) foram analisados por métodos de RT-PCR (reverse transcriptase-polymerase chain reaction), especificamente desenvolvidos para cada gene.

A maioria dos genes respondeu ao tratamento com colesterol, em pelo menos uma das linhas celulares. De salientar, que a resposta ao colesterol, de alguns dos genes estudados foi dependente da concentração de soro no meio. Apenas os níveis de mRNA *SLCO1B1* (OATP-

C), *ABCC3* e *ABCG2* permaneceram inalterados em ambas as linhas celulares, após exposição das células a uma concentração aumentada de colesterol.

Os resultados obtidos revelam um padrão de expressão genética diferencial em modelos celulares de origem hepática ou intestinal e uma resposta diferencial ao colesterol, apontando para diferenças significativas na regulação destes genes nestes tecidos.

Deste modo, neste trabalho são descritos uma variedade de efeitos do colesterol ao nível de genes envolvidos no metabolismo e transporte, em células do fígado e intestino. As nossas observações apontam, em geral, para um aumento da expressão dos transportadores de influxo e de efluxo, assim como para os enzimas metabolizadores de xenobióticos, desencadeada pelo colesterol. No entanto, a maioria dos genes estudados estão descritos como sendo reprimidos pelos ácidos biliares, de onde duas hipóteses podem ser postuladas:

a) O colesterol actua *in vivo* regulando estes genes via a sua conversão a ácidos biliares. Esta hipótese pode explicar as diferenças observadas, uma vez que no fígado de rato, quase todo o colesterol é metabolizado a ácidos biliares, os quais em níveis elevados na célula podem reprimir genes alvo, enquanto nas HepG2 o metabolismo do colesterol pode estar reduzido encontrando-se os ácidos biliares presentes em níveis mais baixos;

b) As alterações mediadas pelo colesterol podem ocorrer ao nível transcripcional através dos próprios metabolitos do colesterol, os quais podem activar os receptores nucleares que regulam a expressão dos transportadores de influxo e de efluxo e os enzimas metabolizadores de xenobióticos. Os oxiesteróis são precursores dos ácidos biliares e podem ligar-se a receptores nucleares como FXR, LXR, PXR e CAR activando os mecanismos reguladores que asseguram a manutenção do balanço de ácidos biliares.

Pode assim colocar-se a hipótese de que, para cada gene analisado, irá ocorrer um cenário diferente, dependendo dos elementos de resposta ao DNA presentes nas regiões reguladoras, que são sítios de ligação para esses factores de transcrição.

No seu conjunto, os resultados obtidos neste estudo contribuem para um melhor conhecimento do metabolismo e transporte de xenobióticos numa situação de hipercolesterolémia, assim como para a compreensão da variabilidade farmacogenética dos genes envolvidos nestas vias.

Dada a importância já demonstrada dos efeitos dos polimorfismos e das alterações na regulação da transcrição dos genes, esta linha de investigação deve continuar a ser explorada. Quando os níveis de diversidade farmacogenética são elevados, como se verificou neste trabalho, este estudo genético assume ainda maior importância, porque a eficácia do tratamento pode estar dependente desta análise. A elucidação dos mecanismos moleculares e o modo como os polimorfismos podem afectar a actividade dos enzimas e transportadores envolvidos no metabolismo do colesterol e dos ácidos biliares pode ajudar a encontrar novos compostos terapêuticos, mais seguros e eficazes, juntamente com dosagens ajustadas ao perfil genético de cada paciente. Este é o objectivo da farmacogenética, optimizar o tratamento,

com selecção do fármaco e ajuste da dosagem adaptada ao perfil genético de cada paciente. Para alcançar este objectivo, é necessário continuar o estudo intensivo das variantes genéticas e o modo como elas afectam as vias metabólicas, nas quais as proteínas polimórficas estão envolvidas. Além disso, também é necessária a análise genética de indivíduos de origens geográficas distintas, para estabelecer diferenças existentes entre padrões genéticos étnico-dependentes.

Por outro lado, os enzimas metabolizadores de xenobióticos juntamente com os transportadores de influxo e de efluxo são reguladores muito importantes no fluxo de ácidos biliares na circulação entero-hepática, podendo funcionar como moduladores da regulação metabólica e da sinalização de ácidos biliares. Assim, é importante compreender como ocorre a regulação destes enzimas e transportadores, a sua relação com o colesterol e os ácidos biliares e ainda, o seu papel no desenvolvimento de doenças. Deste ponto de vista, torna-se ainda importante compreender como é que os compostos terapêuticos interagem com estes enzimas e transportadores e como é que afectam o transporte de ácidos biliares em modelos animais e em pacientes.
Os genes que apresentaram uma maior resposta à presença de colesterol, devem também ser estudados ao nível molecular, assim como o mecanismo de acção de um determinado regulador, o que pode ser feito através da caracterização das interacções DNA/proteína. Este procedimento irá permitir caracterizar os mecanismos moleculares subjacentes ao efeito do colesterol e dos ácidos biliares na expressão de genes.

Palavras-chave:

Citocromos P450 (CYP),

Transportadoes de influxo (SLC),

Transportadores de efluxo (ABC),

Colesterol,

Farmacogenética

Abbreviations

Abbreviations

ABC	adenosine triphosphate- binding cassette
ACAT	acyl-coenzyme A:cholesterol acyltransferase
APO	Apolipoprotein
ATP	adenosine 5'-triphosphate
BCA	bicinchoninic acid
BCRP	breast cancer resistance protein
bp	base pair
BSA	bovine serum albumin
BSEP	bile salt export pump
BSP	Bromosulphthalein
Caco2	human colorectal adenocarcinoma cell line
CAR	constitutive androstane receptor
CBAS3	congenital bile acid synthesis defect type 3
cDNA	complementary deoxyribonucleic acid
CEBP	CCAAT/enhancer-binding protein
CETP	cholesteryl ester-transfer protein
CYP	cytochrome P450
CYP3A4	taurochenodeoxy cholate 6-alpha-hydroxylase*
CYP7A1	cholesterol 7α-hydroxylase
CYP7B1	25-hydroxycholesterol 7-alpha monooxygenase
CYP8B1	sterol 12α-hydroxylase
CYP27A1	sterol 27-hydroxylase
CYP39A1	24-hydroxycholestrol 7-alpha hydroxylase
CYP51A1	sterol 14- demethylase
DHEA	3-beta-Hydroxy-5-androsten-17-one
DIDS	4,4-diisothiocyanostilbene-2,2'-disulfonic acid
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EET	epoxyeicosatrienoic acid
EGTA	ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FBS	fetal bovine serum
FXR	farnesol X receptor
GR	glucocorticoid receptor
HDL	high density lipoprotein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate

HepG2	Human hepatocellular carcinoma cell line
HETE	hydroxyeicosatetraenoic acid
HMG CoA	3-hydroxy-3-methylglutaryl Coenzyme A
HNF	hepatocyte nuclear factor
IDL	intermediate density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LRH	liver receptor homolog
LXR	liver X receptor
MDR	multiple drug resistance
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
MRP	multidrug resistance protein
NADPH	nicotinamide adenine dinucleotide phosphate – reduced form
NTCP	sodium taurocholate cotransporting polypeptide
OAT	organic anion transporter
OATP	organic anion transporting polypeptide
OCT	organic cation transporter
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction- restriction fragment length polymorphism
PGC	peroxisome proliferator-activated receptor-γ coactivator
PhIP	2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine
PPAR	peroxisome proliferator-activated receptor
PVDF	Polyvinylidene fluoride
PXR	pregnane X receptor
RAR	retinoic acid receptor
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
RXR	retinoic X receptor
SDS	sodium dodecyl sulfate
SHP	short heterodimer partner
SLC	solute carrier
SPG5A	spastic paraplegia autosomal recessive type 5A
SRBI	scavenger receptor BI
SREBP	sterol regulatory element-binding protein
STAT	Signal Transducers and Activator of Transcription
VDR	1,25-dihydroxyvitamin D3 receptor
VLDL	very low density lipoprotein

** although this enzyme is active on a plethora of substrates, this is the major activity of this enzyme in the context of bile acid metabolism*

Chapter I - Introduction

1. General Introduction

Cardiovascular diseases are the major causes of death and morbidity in industrialized countries and result in a huge financial burden on the economy. In Portugal, it is the leading cause of mortality (http://www.dgsaude.min-saude.pt/pns/vol2_225.html).

Some risk factors for cardiovascular diseases include high blood pressure, obesity, high cholesterol, diabetes, stress and smoking. In the last two decades, a large effort in research in this area has led to the identification of several potential contributors for the molecular basis of cardiovascular related pathologies.

The prevention of cardiovascular diseases is critically dependent on lipid-lowering therapy. Nowadays there are several kinds of drugs available for this purpose, such as statins, cholesterol absorption inhibitors, bile acid resins, fibrates and nicotinic acid that act by reducing the levels of cholesterol through different pathways (Schmitz, 2005). However, the response to these drugs is highly variable and pharmacogenetic diversity in drug targets may in part contribute to this phenomenon. Therapeutic efficacy and safety may also depend on non-genetic factors that determine the individual levels of the metabolizing enzymes and transporters that act on cardiovascular drugs.

The metabolic system that mediates drug response can be subdivided into three phases. Phases I and II can be envisaged as a complex of metabolic enzymes that generally increase the solubility of the xenobiotics, facilitating their elimination. Phase III corresponds to a number of membrane transport proteins that are responsible for the uptake of compounds to cell and for the efflux of these compounds or their metabolites from the cell. This enzyme complex corresponds mostly to membrane bound (e.g. cytochrome P450s) and cytoplasmic (e.g. glutathione S-transferases) enzymes, associated to a number of membrane transporter proteins comprising influx transporters (SLC - Solute Carrier Transporters) and members of the ATP-binding cassette (ABC) superfamily.

Besides mediating drug response, this metabolic system is also involved in the metabolism of endogenous compounds, such as steroid hormones, bile acids or fatty acids, and as such may be associated with individual risk for disease. On the other hand, the study of the changes that occur in the activity of these enzymes and transporters in response to alterations of the steady state concentrations of endogenous compounds involved in the development of disease can be extremely important, not only to obtain the adequate knowledge of the pharmacokinetics of the drug but also to predict potential adverse drug interactions.

Little is known about the involvement of the detoxification system in the etiology of cardiovascular diseases. The liver, the main detoxification organ in the body, extracts endogenous compounds, as well as drugs from the blood via specialized membrane influx transporters, making them available for biotransformation by metabolizing enzymes.

CYP isoenzymes participate in the mechanisms that control cholesterol homeostasis, catalyzing key steps in cholesterol metabolism, either in the synthesis or in the elimination through conversion to metabolites such as bile acids (Wilson *et al*, 2001). Besides the role of CYPs in

the homeostasis of endogenous substrates, these enzymes may be of crucial importance in the metabolism of drugs used to treat cardiovascular diseases (Siest *et al*, 2003).

An active transport of several compounds is mediated by ABC transporters, which control the absorption and elimination. ABC transporters have been implicated in bile acid, phospholipids and sterol transport, in which the expression of these transporters is itself controlled by lipids (Borst *el al*, 2000).

All the components for this metabolic system are members of different multigenic families, coding for proteins with a wide diversity of substrates. Overall, this metabolic system comprises a high number of different components. Due to their metabolic relationship, the issue of a possible regulatory cross talk is raised, in particular concerning the modulation at the level of gene transcription by nuclear receptors.

The group of orphan nuclear receptors (eg.,LXR, FXR, PXR, CAR and PPAR) appears to play an important role in transcriptional regulation of several ABC transporters and CYP enzymes. These receptors are activated by a wide diversity of ligands, such as bile salts and oxysterols (metabolites of cholesterol), being key regulators in lipid metabolism (Kliewer *et al*, 2002; Pelton *et al*, 2005).

Therefore, and because ABC genes are prone to be involved in human genetic disorders, ABC transporters and transcriptional regulators are promising target molecules for the treatment of lipid disorders.

There is increasing awareness that the genes that code for these enzymes, transporters and transcriptional regulators are highly polymorphic raising the possibility that these individual genetic variations might act as susceptibility factors for cardiovascular diseases. The knowledge on pharmacogenetic markers for cardiovascular diseases as well as on the molecular mechanisms of regulation of the genes involved, may contribute for better surveillance and prevention strategies, as well as for the identification of key regulators potentially useful as therapeutical targets.

2. Cholesterol function and metabolism in mammals

Cholesterol is an isoprenoid molecule with 4 hydrocarbon rings and a hydrocarbon side chain at C17. It contains 27 carbon atoms and possesses a hydroxyl group at C3 (Figure 1).

Cholesterol is a very hydrophobic molecule, with only limited polarity due to the hydroxyl group. Its hydrophobicity is on one hand responsible for its beneficial property to control cell membrane fluidity; and on the other hand makes it very difficult to handle in the aqueous environment of the body, both within cells and between cells. Therefore, sophisticated mechanisms exist to transport cholesterol to its various destinations.

Most of the cholesterol present in the body is a component of cell membranes. As the fatty acid chains of the phospholipids, the molecule of cholesterol is oriented with the polar hydroxyl group close to phospholipids head group. The amount of cholesterol is important to the membrane fluidity, conferring permeability and stability.

Cholesterol is also important, as a substrate for the synthesis of steroid hormones and bile acids as the precursor for a cascade of mediators affecting both genomic and non-genomic processes, as a regulator of gene transcription, protein degradation and enzyme activity and as a direct modulator of the functions of certain plasma membrane proteins (Handschin, 2002).

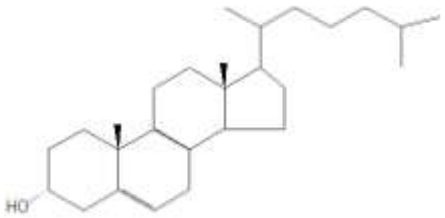

Fig. 1- Structure of cholesterol

2.1. Cholesterol synthesis

Cholesterol synthesis occurs in the cytoplasm and microsomes from the two-carbon acetate group of acetyl-CoA via the mevalonate pathway.

Synthesis starts with the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) from acetyl-CoA and acetoacetyl-CoA. HMG-CoA is subsequently reduced to mevalonate (Figure 2). This rate controlling, irreversible step of cholesterol synthesis is catalysed by the enzyme HMG-CoA reductase. The following synthesis steps include, amongst others, conversion of mevalonate to isopentenyl pyrophosphate, condensation to squalene, and finally cyclization of squalene to form lanosterol. Lanosterol is the precursor of cholesterol (Goldstein and Brown, 1990).

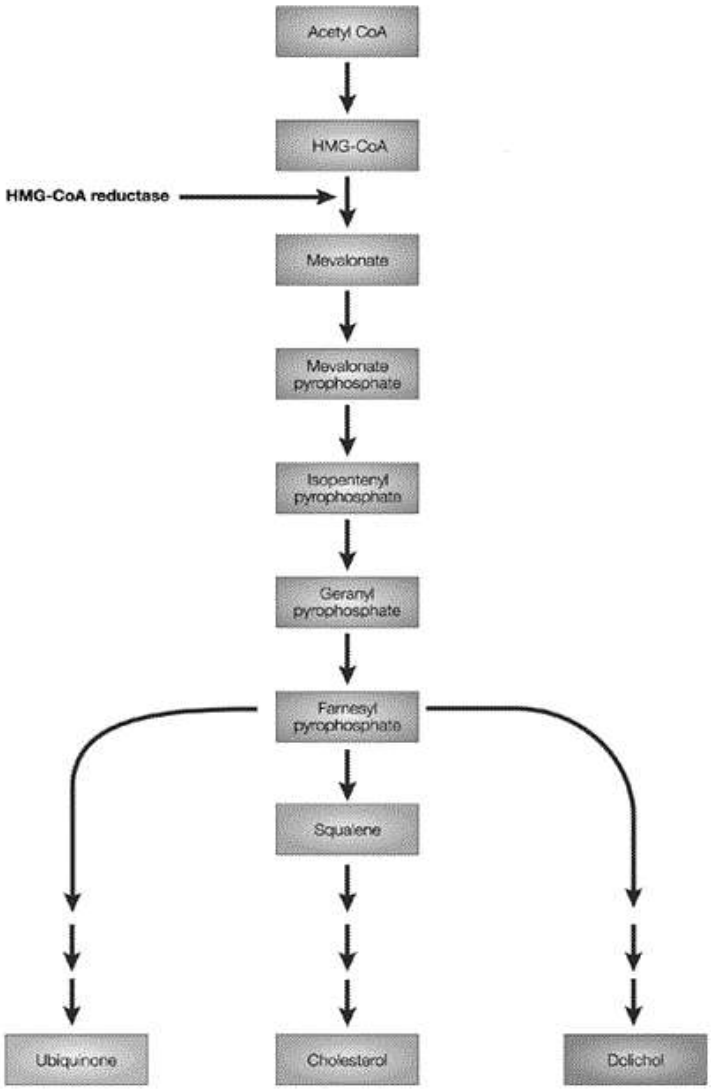

Fig. 2- Synthesis of cholesterol (Tobert, 2003)

Cholesterol is then incorporated into cell membranes, stored as cholesteryl ester or used as substrate for downstream reactions. The largest part of the cholesterol used for downstream reactions is eventually transformed into bile salts. A quantitatively less important part is used for the synthesis of steroid hormones.

2.2. Bile acid synthesis

The hepatic conversion of cholesterol to bile acids represents the major pathway through which cholesterol is eliminated from the body (Andreou and Prokipcak, 1998). Bile acids are important regulators of cholesterol homeostasis by inhibiting hepatic cholesterol metabolism into bile acids or by enhancing uptake of dietary cholesterol. Thus, the levels of bile acids and cholesterol are

linked and tightly controlled by nuclear receptor regulation of both cholesterol catabolism and absorption (Handschin *et al*, 2002).

Bile acids are amphipathic derivatives of cholesterol synthesized exclusively in the liver and stored in the gallbladder from where they are released into the small intestine to form micelles with hydrophobic compounds as fatty acids, sterols and vitamins being absorbed from the jejunum and ileum and recycled via the portal venous system. As a result, only a small portion of bile acids is excreted into urine and feces and the constant bile acids pool is maintained by newly synthesized bile acids (Bahar and Stolz, 1999).

Bile acids can be synthesized from cholesterol by two different ways - the neutral or acidic pathways via a cascade of enzymatic reactions (Figure 3). The neutral pathway, also known as classical pathway results in the formation of cholic acid and the acidic or alternative pathway leads to the synthesis of chenodeoxycholic acid (Inoue *et al*, 2006).

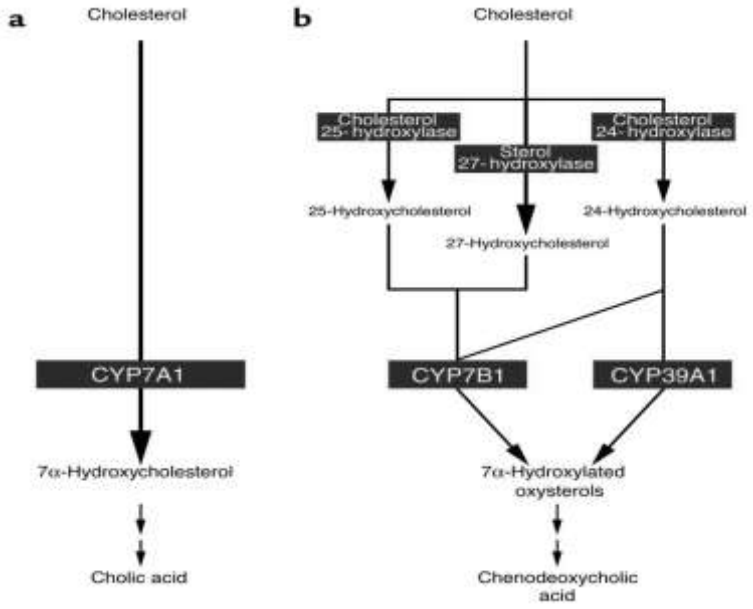

Fig.3- Synthesis of bile acids: **a**) Classical pathway; **b**) Alternative pathway (Beigneux *et al*, 2002)

The classical pathway begins with cholesterol 7α-hydroxylation, a reaction catalyzed by the rate-limiting endoplasmic reticulum enzyme CYP7A1. The resulting 7-hydroxycholesterol is 12-hydroxylated by microsomal CYP8B1 (Pikuleva, 2006). A cascade of downstream reactions ensues, including hydroxylation of the aliphatic side chain at position 27 via mitochondrial CYP27 (Figure 3). In the alternative pathway, 7-hydroxylation is preceded by the formation of several different oxysterols. Although 25- and 27-hydroxycholesterol are subsequently 7-hydroxylated by CYP7B1, the oxysterol 24-hydroxycholesterol is a substrate of CYP39A1, being also substrate of CYP7A1 (Kullak-Ublick *et al*, 2004). In humans, the classical bile acid synthetic pathway is predominant, with only about 10% of bile acids being produced via the alternative pathway. This explains why 50% of the bile acid pool consists of cholic acid and further 20% of its metabolite deoxycholic acid, whereas chenodeoxycholic acid constitutes 30% of the total bile acid pool in humans (Kullak-Ublick *et al*, 1995).

3. Proteins involved in the metabolism and transport of endogenous and exogenous compounds

3.1. Solute carriers (SLC)

The members of the solute carrier (SLC) superfamily are membrane-associated transporters that facilitate the passage of solutes, including peptides, bile acids and drugs across cell membranes in epithelial tissues, such as the liver and the intestine (Hediger *et al*, 2004). SLC transporters play a critical role in a variety of cellular physiological processes, e.g., importing or exporting neurotransmitters, nutrients, or metabolites. SLC transporters are also important in drug absorption, distribution and elimination, thus determining the pharmacokinetic characteristics of many drugs (Meier *et al*, 2007).

The SLC series comprises passive transporters, ion coupled transporters and exchangers (Figure 4). The human SLCs belong to 55 families (He *et al*, 2009) and are expressed in a polarized manner, either at the apical or basolateral membranes. A transporter is assigned to a specific SLC family if it has at least 20-25% amino acid sequence identity to other members of that family (Hediger *et al*, 2004).

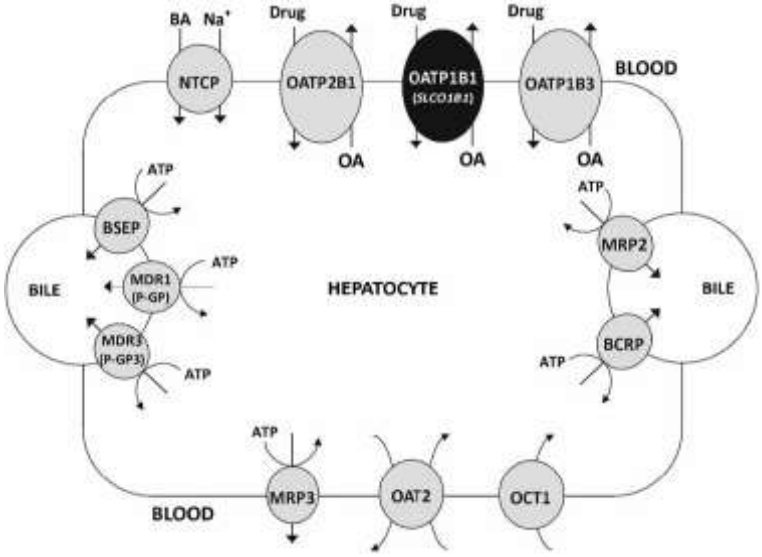

Fig. 4- Scheme representing some of the transporters expressed in the hepatocyte, including the major SLC transporters (Romaine *et al*, 2009)

It is generally assumed that at least 5% (>2000) of all human genes are transport-related, consistent with the biological significance of transporters and their roles in cell homeostasis.

The SLC families represent a considerable portion of these genes: about 319 different SLC human transporter genes exist (Geyer *et al*, 2006).

SLC transporter gene defects have been identified and associated to human diseases (Klaassen and Aleksunes, 2010) and of great importance from a pharmaceutical perspective. SLC activity or expression levels may determine the degree of liver uptake and intestinal absorption of their substrates.

In the present work seven SLC transporters potentially related with the cholesterol homeostasis were studied (Table 1).

Table 1- The human SLC transporters studied in this work

Gene name	Protein name	Major substrates	Tissue distribution	Gene locus	NCBI Sequence accession ID
SLC10A1	NTCP	bile salts	liver, pancreas	14q24.1	NM_003049.2
SLC22A1	OCT-1	acetylcholine, corticosterone	liver, intestine	6q26	NM_003057.2
SLC22A7	OAT2	progesterone, prostaglandins	liver, kidney	6p21.2-p21.1	NM_006672.3
SLCO1A2	OATP-A	eicosanoids, steroidal compounds	liver, brain	12p12	NM_021094.3
SLCO1B1	OATP-C	bile salts, bilirrubin, estrone-3-sulfate	liver	12p12.2	NM_006446.3
SLCO1B3	OATP-8	bile salts, bilirrubin, estrone-3-sulfate	liver	12p12	NM_019844.2
SLCO2B1	OATP-B	estrone-3-sulfate	liver, intestine	11q13	NM_007256.3

3.1.1. SLC10 family

The SLC10 family of sodium/bile salt cotransporters comprises two well-characterized members (NTCP and ASBT), three orphan transporters (P3, P4 and P5) and the sodium-dependent organic anion transporter (SOAT).

The human NTCP (sodium/taurocholate co-transporting polypeptide) is a 349 amino acid membrane glycoprotein with an apparent molecular mass of 56kDa (Hagenbuch and Dawson, 2004). Hallen *et al* (2002) found that NTCP contains an exoplasmic N terminus and a cytoplasmic C terminus. Alanine insertion experiments confirmed that the transmembrane domains have secondary structures and transport activity sensitive to positional displacement, two of these domains critical being for intermolecular interactions and for proper trafficking of NTCP to the plasma membrane. Computer modeling suggests the existence of seven transmembrane domains while experimental evidence is compatible with a nine transmembrane domain model (Mareninova *et al*, 2005). NTCP is expressed at the basolateral (sinusoidal) membrane of human hepatocytes and it is also found at the luminal (apical) membrane of pancreatic acinar cells (Hagenbuch and Dawson, 2004).

NTCP seems to account for most, if not all, sodium-coupled uptake of taurocholate and other bile acids with a Na^+:taurocholate stoichiometry of 2:1 (Geyer *et al*, 2006). It has been shown that NTCP also transports estrogen conjugates such as estrone-3-sulfate as well as bromosulphthalein (BSP), 3-beta-Hydroxy-5-androsten-17-one (DHEAs) and thyroid hormones (Koesters and Karpen, 2008). *In vitro* studies demonstrated that NTCP transports drugs covalently bound to taurocholate and also chenodeoxycholate-3-sulfate and taurolithocholate-3-sulfate (Kullak-Ublick *et al*, 1997; Hagenbuch and Meier, 1996; Hata *et al*, 2003; Pauli-Magnus *et al*, 2005; Geyer *et al*, 2006; Pauli-Magnus and Meier, 2006). Some drugs, as cyclosporine A, R- and S-propranolol, BSP, furosemide, 4,4-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), 17-b-estradiol-3-sulfate, and tauro-lithocholate-3-sulfate, were identified as inhibitors of NTCP-mediated transport (Kim *et al*, 1999; Kramer *et al*, 1999; Hata *et al*, 2003).

The human *SLC10A1* gene is located on chromosome 14 at 14q24 on the reverse strand (Hagenbuch and Meier, 1994), contains 5 exons and spans about 23kb. The promoter has no consensus TATA or CAAT box sequence. Shiao *et al* (2000) identified a number of putative DNA-binding sites for the liver-enriched binding factors hepatocyte nuclear factor 3 (HNF3), HNF6 and CCAAT/enhancer-binding proteins (CEBP), as well as binding sites for numerous ubiquitous transcription factors. The promoter also contains potential sites for Signal Transducers and Activator of Transcription (STAT) proteins. Transcription produces four different mRNAs, two alternatively spliced variants that give rise to two functional proteins and two unspliced forms giving rise to two non-functional proteins. 1216bp of this gene are antisense to spliced gene SFRS5, raising the possibility of regulated alternate expression (Shiao *et al*, 2000). *SLC10A1* expression was shown to be regulated by a complex interplay of several ligand-activated receptors (retinoic acid receptor α (RAR), glucocorticoid receptor (GR)) and hepatic transcription factors (hepatocyte nuclear factors HNF1α, HNF4α and HNF3α, members of the CEBP family and small heterodimer partner SHP-1) (Geyer *et al*, 2006; Shiao *et al*, 2000; Jung *et al*, 2004). SLC10A1 regulation by the GR occurs by a ligand dependent manner. This activation by GR is blocked by Farnesoid X receptor (FXR) induced expression of short heterodimer partner (SHP), providing a negative feedback mechanism by bile acids on their uptake, while activation of NTCP by GR was increased in the presence of the coactivator Peroxisome proliferator-activated receptor-γ coactivator (PGC-1) (Eloranta *et al*, 2006). Regulation by FXR occurs by dimerization with retinoic X receptor (RXR) followed by activation of its target, SHP.

There are no reports of association between disease and defects in *SLC10A1*. It has been suggested that *SLC10A1* polymorphism may play an important role in the development of hypercholesterolemia, but there are no conclusive studies published till now. Some polymorphisms in this gene were described as ethnicity dependent and related with variations in NTCP mRNA expression levels. These variants are generally found at low frequencies and were associated with reduced membrane localization and with the decreased to near absence of transport activity of bile acids (Kullak-Ublick et al, 1997; Ho et al, 2004). However the

evidence available is limited to subsets of the American population, with variable ancestries, with no known report on native populations.

3.1.2. SLC22 family

Most transporters of the SLC22 family are polyspecific, transporting multiple different substrates, and are subdivided into three groups: organic cation transporters (OCTs), organic anion transporters (OATs) and organic zwitterions/cation transporters (OCTNs) (Koepsell and Endou, 2004).

The human OCT-1 is a 554 amino acid membrane protein with a molecular mass of 61kDa and OAT2 is a 548 amino acid membrane protein with a molecular mass of 60kDa. Both transporters contain twelve putative transmembrane domains and are plasma membrane integral proteins with transporter-specific motifs (Simonson *et al*, 1994) and they are mainly expressed in the liver, at the basolateral membrane of the hepatocytes. Their expression was also found in kidney, heart, skeletal muscle and intestine but at lower levels (Katsura and Inui, 2003).

OCT-1 is the main organic cation uptake system in hepatocytes. This protein translocates organic cations in an electrogenic and pH-dependent manner across the membrane in both directions.

OCT-1 mediates the transport of a broad array of organic cations including therapeutically important compounds such as desipramine, aciclovir, ganciclovir and metformin (Koepsell and Endou, 2004; Mizuno *et al*, 2003) and it is also involved in the transport of endogenous compounds such as choline, guanidine, histamine, epinephrine, norepinephrine and dopamine (Wessler *et al*, 2001). The transport of organic cations is inhibited by a broad array of compounds such as cocaine, atropine, guanidine and choline.

OAT2 is involved in the sodium-independent multispecific organic anion transport. It mediates the uptake of small hydrophilic organic anions such as prostaglandins, hormones and other endogenous compounds, antibiotics, antivirals, H2 antagonists, cytostatics, diuretics, non-steroidal anti-inflamatory drug (NSAIDs) and statins (Robertson and Rankin, 2006). OAT2 plays an important role in distribution and excretion of many drugs, working as anion exchanger, by coupling the uptake of an organic anion into the cell to the release of another organic anion from the cell. This transport is made utilizing the gradient of anions, such as α-ketoglutarate, lactate and nicotinate to drive uphill uptake of organic anions against the inside negative membrane potential (Rizwan and Burckhardt, 2007).

Both human genes are located on chromosome 6 on the direct strand, *SLC22A1* at 6q26 and *SLC22A7* at 6p21.2-p21.1 (Koehler *et al*, 1997). *SLC22A1* covers 37.14kb and has 7 exons (Hayer *et al*, 1999), while *SLC22A7* covers 10.47kb and has 10 exons (Kok *et al*, 2000). Two transcript variants encoding two different isoforms of OCT-1 have been found for *SLC22A1* gene, but only the longer variant encodes a functional protein. The *SLC22A7* gene includes a

734bp antisense sequence to spliced gene ZNF318 and CRIP3, raising the possibility of regulated alternate expression, but all the variants spliced and unspliced encode functional proteins, altogether 9 different isoforms.

SLC22A1 expression is highly regulated (Koepsell and Endou, 2004) but little is known about the gene regulation of *SLC22A7*. HNF-4 was described to transactivate *SLC22A1* and *SLC22A7* promoters. In the proposed mechanism bile acids negatively target the HNF-4 mediated activation through FXR-dependent mechanisms, resulting in decreased expression of these transporters in conditions associated with elevated intrahepatic concentrations of bile acids (Popowski *et al*, 2005).

There are no reports of association between disease and defects in *SLC22A1* or *SLC22A7*. Shu *et al* (2003) functionally characterized 15 protein-altering variants of OCT-1, where the variants with reduced or eliminated function had amino acid substitutions that resulted in more radical chemical changes and were less evolutionary favourable. Shu *et al* (2007) investigated the role of OCT-1 in the therapeutic effects of the drug metformin, an anti-diabetic drug for which an extremely variable response is observed. Glucose tolerance tests in healthy volunteers showed significantly lower effects of metformin in individuals carrying reduced-function *SLC22A1* polymorphisms, indicating that OCT-1 is important for metformin therapeutic action and that genetic variation in this gene may contribute to variation in response to the drug.

3.1.3. SLCO family

The SLCO family consists of sodium independent organic anion transporting polypetides (OATPs), most of which have broad substrate specificity (Hagenbuch and Meier, 2004). While many OATPs play vital roles in liver, some of them also have important functions in the intestine.

Their substrates include hormones and their conjugates, eicosanoids, some steroidal compounds, several drugs such as pravastatin and endogenous conjugated and unconjugated bile acids such as taurocholate (Abe *et al*, 1999; Konig *et al*, 2000a; Tamai *et al*, 2000; St-Pierre *et al*, 2002; Wang *et al*, 2003b).

The human OATP-A is a 670 amino acid membrane protein (Kullak-Ublick *et al*, 1995) and OATP-C is a 691 amino acid membrane glycoprotein. Both transporters produce two different isoforms, one glycosylated and another unglycosylated. The human OATP-8 and OATP-B are 702 amino acid and 709 amino acid, respectively, membrane glycoproteins, both with molecular mass of 77kDa (Nagase *et al*, 1998). They all have 12 putative transmembrane domains and several potential N-linked glycosylation sites (Konig *et al*, 2000a, Tamai et al, 2000). OATP-8 shares 80% sequence identity with OATP-C (Konig *et al*, 2000b). These OATP transporters are expressed on the basolateral membrane of the hepatocytes and OATP-A and OATP-B are also expressed in several other tissues including liver, pancreas, lung, ovary, testis, gut and spleen (Kullak-Ublick *et al*, 1995; Tamai *et al*, 2000). The extrahepatic expression of OATP-A suggests

a general role in transepithelial organic anion transport, while OATP-C which is highly expressed in liver may play an important role in clearance of organic anions from this organ. OATP-8 expression was not detected in other tissues, but this transporter is highly expressed in some cancer cell lines derived from colon, pancreas, liver and gall bladder.

OATP-A acts as anion exchanger. It has low affinity for bile acids compared with the other OATP family members but high affinity for the steroid precursor DHEAS (St-Pierre *et al*, 2001), indicating a minor role in bile acid transport. OATP-C represents an important Na^+-independent bile salt uptake system in human liver (St-Pierre *et al*, 2001), but transports taurocholate with lower affinity than NTCP.

SLCO1A2/1B1/1B3 genes are located on chromosome 12 while *SLCO2B1* is located on chromosome 11. *SLCO1A2* is at 12p12 (Kullak-Ublick *et al*, 1995) on the reverse strand. It covers 185.54kb and has 14 exons. 104pb of this gene are antisense to spliced gene *FLJ22028*, raising the possibility of regulated alternate expression. Alternate splicing of *SLCO1A2* results in three transcript variants encoding two different isoforms. *SLCO1B1* gene is at 12p12.2 on the direct strand. It covers 29.10kb and has 14 exons. *SLCO1B3* gene is at 12p12 on the direct strand. It covers 106kb and has 14 exons (Konig *et al*, 2000). The human *SLCO2B1* gene is at 11q13 on the direct strand. It covers 105.99kb and has 14 exons (Nagase *et al*, 1998).

The expression of the OATP family is controlled by FXR, and it is suggested that expression declines in situations of biliary congestion (Mikkaichi *et al*, 2004). The activation of FXR decreases the expression level of *SLCO1B1* and *SLCO1B3*. This action is mediated by SHP-1 and inhibits the function of HNF-1α which is a main transcription factor in the regulation of these transporters (Faber *et al*, 2003; Mikkaichi, 2004), which are also down-regulated in cholestasis (Faber *et al*, 2003).

SLCO1A2 expression has been shown in human breast carcinoma and in breast cancer cell lines, where this transporter is suspected to be involved in estrone-3-sulfate transport (Nozawa *et al*, 2005).

Some polymorphisms have been described as ethnic-dependent and were related with changes in transport activity, indicating a possible role for *SLCO1A2* in inter-individual variability in drug disposition (Lee et al, 2005). However, the evidence was limited to American subjects with different ethnic backgrounds. There are no reports of association between disease and defects in *SLCO1B1* and *SLCO1B3*, apart from a role for *SLCO1B1* SNPs in total serum bilirubin levels (Johnson *et al*, 2009). Michalski *et al* (2002) demonstrated that two amino acid changes N130D and P155T, in *SLCO1B1*, led to altered substrate specificity compared with the most common variant. Another polymorphism, a rare mutation, L193R lead to a weaker expression at the protein level and showed altered cellular distribution, being retained intracellularly. Takane *et al* (2006) analysed the response of hypercholesterolemic patients to the cholesterol-lowering drug pravastatin and the carriers of the *15 haplotype (388A>G and 521T>C) had significantly smaller reductions in total low density lipoprotein (LDL) cholesterol than non-carriers at 8 weeks of treatment, although there were no significant differences at 1 year post-treatment, suggesting

a slow response of *SLCO1B1**15 haplotype to pravastatin. The SEARCH Collaborative Group (2008) identified a significant association between common variants in the *SLCO1B1* gene and statin-induced myopathy. Polymorphism in the *SLCO1B3* gene has been extensively studied, and some SNPs have been shown to be tightly associated to response to several drugs, such as lopinavir, docetaxel, mycophenolic acid and rifampin (Hartkoorn *et al*, 2010; Kiyotani *et al,* 2008; Miura *et al*, 2007; Weiner *et al*, 2010). The pharmacogenetics of *SLCO1B1* and *SLCO1B3* has been the topic of several studies. Polymorphisms in these transporters may contribute to the inter-individual variability observed in statins pharmacokinetics and efficacy and may increase the probability of drug-drug interactions.

Polymorphisms in the *SLCO2B1* gene are less intensively studied. However two nonsynonymous SNPs were described, one of them dependent on ethnicity. These SNPs were related with decreased in transport activity without affecting substrate affinity (Zahr *et al*, 2008). Some studies have reported an association between selected SNPs in *SLCO2B1* and response to montelukast (Lima *et al*, 2009) while no association was observed for pravastatin (Niemi *et al*, 2004) and mycophenolic acid (Miura *et al*, 2007).

3.2. Cytochrome P450 (CYP) enzymes

Cytochrome P450 (CYP) enzymes are a group of heme-thiolate proteins with a characteristic absorption maximum at 450nm. P450s can carry out several reactions, such as reduction, dehydrogenation, demethylation and dehydration, however monooxigenase reactions are the most common (Guengerich, 2001).

In humans, these enzymes are all membrane-bound, localizing either in the endoplasmic reticulum or in the inner mitochondrial membrane. While microsomal P450s have both exogenous and endogenous substrates, mitochondrial P450s play a crucial role in the metabolism of endogenous compounds such as steroid hormones, bile acids and fatty acids among others (Guengerich, 2001). In the liver, these enzymes are dependent on a nicotinamide adenine dinucleotide phosphate (NADPH) -dependent electron transport pathway.

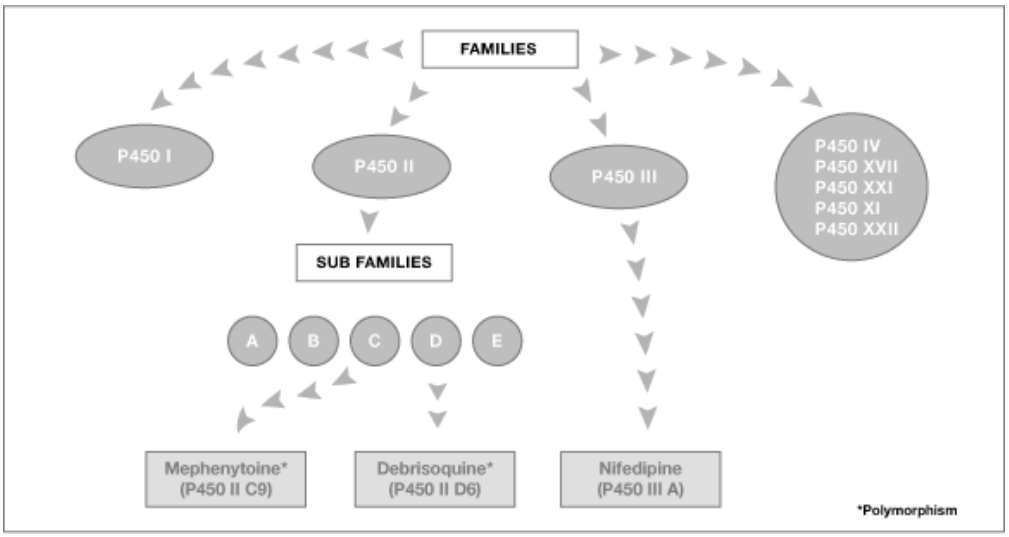

Fig. 5- Cytochrome P450 enzymes (http://www.bioreglab.be/en/bio3.html)

Cytochromes P450 are classified into families and subfamilies according to their amino acid sequence (Figure 5). A sequence homology of 40% defines a family and of 55% a subfamily. There are 57 isoforms of P450s in humans distributed by 18 families and 43 subfamilies. In the present work seven P450 enzymes potentially related with cholesterol metabolism were studied (Table 2).

Table 2- The human cytochrome P450 enzymes studied in this work

Gene name	Protein name	Major substrates	Tissue distribution	Gene locus	NCBI Sequence accession ID
CYP3A4 EC1.14.13.97	Taurochenodeoxy cholate 6-alpha-hydroxylase	taurochenodeoxy cholate, taurodeoxy cholate	liver, prostate	7q21.1	NM_017460.3
CYP7A1 EC1.14.13.17	cholesterol 7alpha-monooxygenase	cholesterol	liver	8q11-q12	NM_000780.3
CYP7B1 EC1.14.13.100	25-hydroxycholesterol 7-alpha monooxygenase	oxysterols	ubiquitous	8q21.3	NM_004820.3
CYP8B1 EC1.14.13.95	7-alpha hydroxycholest-4-en-3-one 12alpha-hydroxylase	7-alpha-hydroxy-4-cholesten-3-one	liver	3p22-p21.3	NM_004391.2
CYP27A1 EC1.14.13.15	cholestanetriol 26-monooxygenase	cholesterol, sterols	ubiquitous	2q33-qter	NM_00784.3
CYP39A1 EC1.14.13.99	24-hydroxycholesterol 7-alpha hydroxylase	(24R)-cholest-5-ene-3-beta, 24-diol	liver	6p21.1-p11.2	NM_016593.3
CYP51A1 EC1.14.13.70	sterol 14- demethylase	steroids with a 14-alpha-methyl group	ubiquitous	7q21.2-q21.3	NM_000786.3

3.2.1. CYP3 family

The CYP3 family, in humans, comprises three isoforms in adults (CYP3A4, CYP3A5 and CYP3A43) and one fetal isoform (CYP3A7) and is responsible for the metabolism of the majority of all drugs.

The human CYP3A4 is a 503 amino acid membrane protein with a molecular mass of 57kDa, localized in the endoplasmic reticulum in liver, intestine, prostate and other organs. It performs a variety of oxidation reactions of structurally unrelated compounds, including steroids, fatty acids and xenobiotics (Shet *et al*, 1993; Markowitz *et al*, 2003) and is involved in the metabolism of approximately half the drugs which are used today, including acetaminophen, codeine, cyclosporine A, diazepam and erythromycin (Lown *et al*, 1997). It is induced by glucocorticoids and by various foreign compounds as drugs, pesticides and carcinogens (Wrighton and Stevens, 1992).

The human *CYP3A4* gene is located on chromosome 7 at 7q21.1 on the reverse strand (Inoue *et al*, 1992). It covers 27.34kb and comprises 13 exons. *CYP3A4* exhibits adaptive transcriptional regulation in response to a range of xenobiotics including some therapeutic drugs, via members of the nuclear receptor superfamily that function as ligand-activated transcription factors, such as the pregnane X receptor (PXR), constitutive androstane receptor (CAR) and 1,25-dihydroxyvitamin D receptor (VDR) (Stedman, 2004). Tirona *et al* (2003) showed that HNF4α is critically involved in the PXR and CAR mediated transcriptional activation of CYP3A4. They identified a specific cis-acting element in the *CYP3A4* gene enhancer that confers HNF4α binding and thereby permits PXR and CAR mediated gene activation.

There are several polymorphisms described for the *CYP3A4* gene, which might potentially affect drug pharmacokinetics and risk for disease (www.cypalleles.ki.se). However, the real clinical significance of *CYP3A4* variants is far from being established. Some variants have been associated with prostate cancer (Rebbeck *et al*, 1998) and leukemia (Felix *et al*, 1998) an effect probably linked to decreased inactivation of testosterone (Rebbeck *et al*, 1998) or of therapeutic drugs, respectively.

3.2.2. CYP7 family

The CYP7 family is composed by 2 subfamilies, each with only one member, CYP7A1 and CYP7B1. These enzymes are responsible for bile acids biosynthesis.

The human CYP7A1 and CYP7B1 are 504 amino acid membrane proteins with a molecular mass of 58kDa. CYP7A1 protein contains putative heme and steroid-binding domains (Noshiro and Okuda, 1990) and CYP7B1 shares 39% sequence identity with CYP7A1. CYP7A1 is a liver-specific enzyme (Mast *et al*, 2005), while CYP7B1 is an ubiquitous protein also expressed in brain, testis, ovary, prostate, colon, kidney and small intestine, being both enzymes expressed at the endoplasmic reticulum.

CYP7A1 has a narrow substrate specificity determined by a tight fit between the CYP7A1 active site and the cholesterol conformation (Mast *et al*, 2005). However, it was shown that CYP7A1 also can metabolize 20S-, 24S, 25- and 27-hydrocholesterol (Norlin *et al*, 2000). CYP7A1 activity is highly variable among healthy individuals, displaying a 5- to 10-fold variation. The activity of this enzyme was reported to be negatively regulated by bile acids but not by cholesterol increase (Chiang, 2004). External factors such as diet, some drugs and hormones, diurnal rhythm, obesity and age also influence CYP7A1 activity (Chiang, 1998). CYP7B1 catalyzes the first reaction in the cholesterol catabolic pathway of extrahepatic tissues, which converts cholesterol to bile acids catalyzing the 7α-hydroxylation of 27- and 25-hydroxycholesterol (Rose *et al*, 1997). CYP7B1 also has as substrates steroids such as DHEA, pregnenolone, 5 α-androstane-3β,17diol, 17β-estradiol and testosterone (Fitzpatrick *et al*, 2001).

The human *CYP7* genes are located on chromosome 8 on the reverse strand, *CYP7A1* at 8q11-q12 and CYP7B1 at 8q21.3. Both genes have 6 exons but *CYP7A1* spans about 10.06kb (Cohen et al, 1992) while *CYP7B1* covers 211.03kb (Setchell *et al*, 1998). Characterization of the 5-prime flanking region revealed the presence of consensus recognition sequences for a number of liver-specific transcription factors. Molowa et al (1992) identified a TATA box and a modified CAAT box in the promoter region of the *CYP7* gene and also demonstrated the presence of a modified sterol response element and three potential recognition sites for HNF3.

Transcription of *CYP7A1* is down regulated by FXR via a complex molecular mechanism that involves the coordinated regulation of several liver-enriched nuclear receptors. Bile acids bind FXR, which dimerizes with RXR and transcriptionally activates its target, SHP. SHP represses *CYP7A1* indirectly through its association with another nuclear receptor, liver receptor homolog-1 (LRH-1) (Lu *et al*, 2000). *CYP7B1* is also regulated by bile acids at the transcriptional level, in rats and mice, and hormones and cholesterol was also suggested to regulate *CYP7B1* (Ren *et al*, 2003).

CYP7A1 seems to be a candidate gene for disorders of cholesterol and bile acid metabolism and some authors suggested that *CYP7A1* may be involved in gallstone disease or in familiar hypertriglyceridemia (Cohen *et al*, 1992; Paumgartner and Sauerbruch, 1991; Angelin *et al*, 1987). Polymorphisms in *CYP7A1* were associated with increased plasma LDL-cholesterol concentrations (Wang *et al*, 1998). Mutations in *CYP7B1* are related with spastic paraplegia autosomal recessive type 5A (SPG5A), a neurodegenerative disorder characterized by a slow, gradual, progressive weakness and spasticity of the lower limbs (Wilkinson *et al*, 2003; Tsaousidou *et al*, 2008). Absence of CYP7B1 is the cause of congenital bile acid synthesis defect type 3 (CBAS3), which is characterized by severe cholestasis, cirrhosis and liver synthetic failure (Setchell *et al*, 1998).

3.2.3. CYP8 family

The CYP8 family comprises 2 subfamilies, each containing only one member, CYP8A1 and CYP8B1. These enzymes have different functions, with CYP8B1 being involved in bile acids biosynthesis.

Human CYP8B1 is a 501 amino acid membrane protein with a molecular mass of 58kDa. It contains a hydrophobic, membrane-spanning N terminus and conserved oxygen-binding and heme-binding segments (Gafvels *et al*, 1999). It is an endoplasmic reticulum membrane protein expressed in liver, responsible for the conversion of 7-alpha-hydroxy-4-cholesten-3-one into 7-alpha, 12-alpha-dihydroxy-4-cholesten-3-one. It has broad substrate specificity. CYP8B1 is a fundamental enzyme in the synthesis pathway of cholic acid regulating the ratio between this compound and chenodeoxycholic acid, which is normally of 2:1. The activity of CYP8B1 is dependent on circadian rhythm and is also down-regulated by bile acid intake and up-regulated by the bile acid-binding resin - cholestyramine.

The human *CYP8B1* gene is located on chromosome 3 at 3p22-p21.3 on the reverse strand. It is an intronless gene, comprising only 1 exon and spans about 3.97kb. The promoter region of *CYP8B1* contains multiple regulatory motifs, including a possible TATA box 51bp from the transcription start site (Gafvels *et al*, 1999). *CYP8B1* promoter activity is strongly activated by HNF4α and repressed by bile acids (Zhang and Chiang, 2001). Functional analyses determined that SHP represses HNF4α-induced *CYP8B1* transcription, leading Zhang and Chiang (2001) to conclude that bile acids repress human *CYP8B1* transcription by reducing the transactivation activity of HNF4α through the interaction of HNF4α with SHP and a reduction of HNF4α expression in liver.

Although *CYP8B1* can be a target for cholesterol lowering therapy, there are no reports relating polymorphisms with alterations in activity of this enzyme.

3.2.4. CYP27 family

CYP27 family is divided into 3 subfamilies, each comprising one member, CYP27A1, CYP27B1 and CYP27C1. These enzymes have different functions, CYP27A1 being involved in bile acids biosynthesis.

The human CYP27A1 is a 531 amino acids mitochondrial membrane protein with a molecular mass of 60kDa (Cali and Russel, 1991). It is expressed in the liver and in all tissues analyzed so far and it hydroxylates a variety of sterols at the C27 position. In the bile acid synthesis pathway, CYP27A1 catalyzes the first step in the oxidation of the side chain of sterol intermediates. It has also a vitamin D3-25-hydroxylase activity. The CYP27A1-mediated pathway of bile acids biosynthesis is up-regulated when the classical pathway is suppressed. The activity of CYP27A1 is known to be differentially altered by phospholipids *in vitro* (Murtazina *et al*, 2004).

The human *CYP27A1* gene is located on chromosome 2 at 2q33-qter on the direct strand (Cali and Russel, 1991). It has 9 exons and covers 34.29kb (Leitersdorf *et al*, 1993). The putative promoter region was found to be rich in GC residues and to contain potential binding sites for the transcription factor SP1 and the liver transcription factor LF-B1 (Leitersdorf *et al*, 1993). *CYP27A1* is transcriptionally repressed by bile acids, however the response is lower than that of *CYP7A1* (Pikuleva, 2006; St-Pierre *et al*, 2001). One transcription factor identified is HNF1α, whose binding to the *CYP27A1* promoter is reduced by bile acids (St-Pierre *et al*, 2001). The CYP27A1 products are ligands for LXRα and LXRβ, which are involved in transcriptional regulation of cholesterol homeostasis, indicating that CYP27A1 may have a regulatory role (Pikuleva, 2006).

Mutations in *CYP27A1* were identified in patients with cerebrotendinous xanthomatosis, a rare sterol storage disorder characterized clinically by progressive neurologic dysfunction, premature atherosclerosis and cataracts (Guyant-Marechal *et al*, 2005).

3.2.5. CYP39 family

CYP39 family has only one member, CYP39A1, which is involved in the hydroxylation of hydroxycholesterol.

Human CYP39A1 is a 469 amino acid membrane protein with a molecular mass of 54kDa.

It is constitutively expressed in the endoplasmic reticulum in the liver and it is involved in the conversion of cholesterol to bile acids. Its substrates include the oxysterols 25-hydroxycholesterol, 27-hydroxycholesterol and 24-hydroxycholesterol (Li-Hawkins *et al*, 2000).

The human *CYP39A1* gene is located on chromosome 6 at 6p21.1-p11.2 on the reverse strand. It has 12 exons and covers 103.42kb (Li-Hawkins *et al*, 2000). There is no description about gene regulation of *CYP39A1*, in humans. However, in mice the levels of hepatic *CYP39A1* mRNA do not change in response to dietary cholesterol, bile acids and a bile-acid binding resin.

Although there are no published reports of polymorphisms in CYP39A1 or its association with disease, several polymorphisms in the promoter and coding regions can be found in the NCBI SNP Database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?chooseRs=all&go=Go&locusId=9481), whose relevance for enzyme function is still unknown.

3.2.6. CYP51 family

CYP51 family is composed by one gene, CYP51A1, and 3 pseudogenes. CYP51A1 is involved in cholesterol biosynthesis.

The human CYP51A1 is a 503 amino acids endoplasmic reticulum membrane protein with a molecular mass of 57kDa ubiquitously expressed, with high levels in testis, ovary, adrenal,

prostate, liver, kidney and lung and participates in the synthesis of cholesterol, steroids and other lipids. CYP51A1 demethylates lanosterol to cholesterol producing oxysterols, which inhibit hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase and sterol synthesis (Gibbons, 2002). CYP51A1 activity is inhibited by several compounds, azoles (imidazoles and triazoles) being the most broadly known. Inhibition of sterol 14α-demethylase activity blocks sterol biosynthesis, lowering endogenous cholesterol production in animals.

The human *CYP51A1* gene is located on chromosome 7 at 7q21.2-q21.3 on the reverse strand. It has 10 exons and covers 67.52kb (Rozman *et al*, 1996). The human promoter has no TATA or CAAT patterns and has a GC-rich sequence (Rozman *et al*, 1996).

Although there is no description of association between disease and *CYP51A1* mutations, this enzyme may be of great importance as a drug target. *CYP51A1* gene is polymorphic with SNPs in the promoter and coding regions as well as in introns (Leroux *et al*, 2007).

3.3. ATP-binding cassette (ABC) transporters

ABC transporters are a superfamily of transmembrane proteins which mediate the transport of a wide variety of substrates across different cellular membranes, a process driven by the hydrolysis of ATP (Higgins, 1992).

Most mammalian ABC transporters contain two ATP-binding and two transmembrane domains (Figure 6). In the ATP-binding domain the highly conserved Walker A and Walker B motifs are present which are involved in ATP binding and hydrolysis. The transmembrane domain is formed by 6-11 membrane-spanning α-helices. Some ABC-transporters contain only one ATP-binding site and one set of 6 transmembrane helices. They are therefore called “half transporters” and are considered to act as homodimers or as heterodimers with other halftransporters (Borst and Elferink, 2002). All ABC transporters have a distinctive feature, the C motif, with the consensus sequence “LeuSerGlyGlyGln” (Stefková *et al*, 2004).

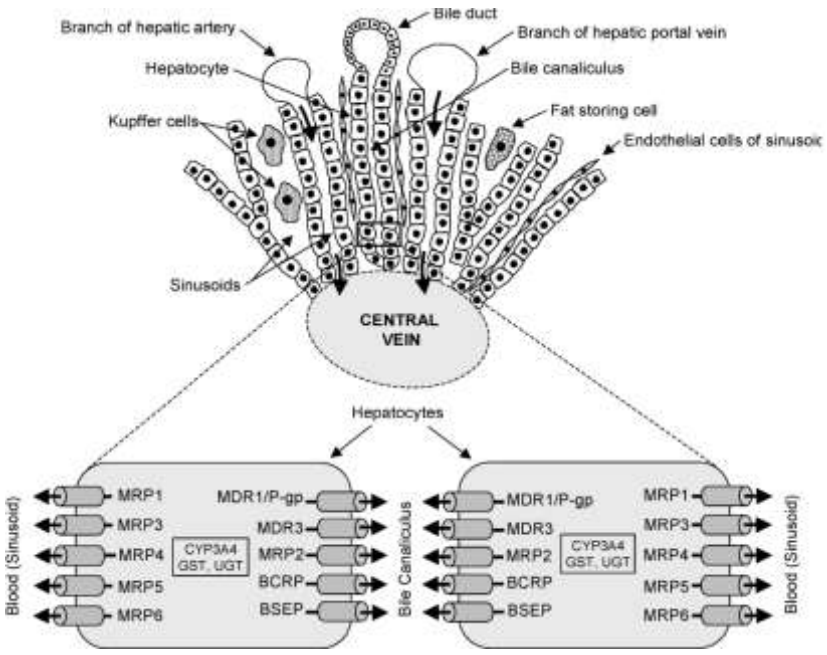

Fig. 6- ATP-binding cassette transporters expressed in the liver (Choudhuri and Klaassen, 2006)

In this superfamily of ABC transporters 48 proteins have been identified up to now (Borst and Elferink, 2002). They are classified in 7 groups (A-G) based on their structure and homology. In this work eight transporters potentially related with the cholesterol homeostasis were studied (Table 3).

Many ABC transporters have been associated to human diseases, and may be of key importance to understand individual cholesterol homeostasis and pathology. These transporters together with SLC transporters and drug metabolizing enzymes may be of great importance in the development of therapeutical strategies as well in the prediction of some genetic conditions.

Table 3- The human ABC transporters studied in this work

Gene name	Protein name	Major substrates	Tissue distribution	Gene locus	NCBI Sequence accession ID
ABCA1	ABC1	phospholipids, cholesterol	ubiquitous	9q31.1	NM_005502
ABCB11	BSEP	conjugated and non-conjugated bile salts	liver	2q24	NM_003742
ABCC2	MRP2	bilirubin glucuronide, leukotriene C4	liver, kidney	10q24	NM_000392
ABCC3	MRP3	sulfated bile salts	liver	17q22	NM_003786
ABCC6	MRP6	glutathione conjugates	liver, kidney	16p13.1	NM_001171
ABCG2	BCRP	cholesterol, estradiol, progesterone	Placenta, liver, intestine	4q22	NM_004827
ABCG5	sterolin	sterols, cholesterol	liver, colon, intestine	2p21	NM_022436
ABCG8	sterolin	sterols, cholesterol	liver, colon, intestine	2p21	NM_022437

3.3.1. ABCA family

The ABCA family is characterized by the presence of some hydrophobic amino acids which span the membrane within the putative regulatory domain. This subfamily comprises 12 members divided into two subgroups according with its intron structure and phylogenetic analysis (Stefková *et al*, 2004).

ABCA transporters have an important role in the organism, being determinant in the development of specific genetic diseases.

Human ABCA1 is a 2261 amino acid integral membrane protein with a molecular mass of 254kDa, comprising two halves of a similar structure, each half containing the Walker A, Walker B and Walker C motifs (Zhao *et al*, 2000).This transporter is predicted to have an N terminus oriented into the cytosol and 2 large extracellular loops that are highly glycosylated and linked by 1 or more cysteine bonds (Bungert *et al*, 2001; Fitzgerald *et al*, 2001).

ABCA1 is widely expressed having been found mainly in placenta, liver, lung, adrenal glands and fetal tissues and among other organs where it is less expressed (Langmann et al, 1999). It is a cAMP-dependent and sulfonylurea-sensitive anion transporter which mediates the transport of cholesterol, phospholipids and other lipophilic molecules across cellular membranes, a key step in cholesterol uptake into high density lipoproteins (HDL) particles (Oram and Heinecke, 2005). ABCA1 is highly regulated posttranscriptionally at the protein stability level (Oram and Vaughan, 2006). The protein is highly unstable and its interaction with apolipoproteins reduces the rate of protein degradation by inhibiting ABCA1 proteolysis by calpain and by activating other signaling events. This feed-back mechanism allows maintaining the ABCA1 levels when acceptors for cellular lipids are available (Wang *et al*, 2003a; Arakawa and Yokoyama, 2002).

The human *ABCA1* gene is localized on chromosome 9 at 9q31.1 on the reverse strand (Luciani *et al*, 1994). It contains 50 exons and spans about 149kb (Santamarina-Fojo *et al*, 2002). Pullinger et al (2000) analyzed the promoter region of *ABCA1* and identified seven putative SP1-binding sites, four sterol regulatory elements (SREs) similar to the low density lipoprotein receptor (LDLR) promoter region, a CpG island, a possible weak TATA box, two distal CCAAT sequences and binding sites for several other transcription factors. Expression of *ABCA1* is highly induced by cholesterol. This process occurs through the action of nuclear receptors, particularly by LXR, which forms an obligate heterodimer with RXR (Costet *et al*, 2000; Repa *et al*, 2000). LXR binds to response elements localized in the promoter region and in the first intron of the *ABCA1* gene.

Mutations in *ABCA1* have been associated high-density lipoprotein deficiency type 1 (HDLD1) and type 2 (HDLD2). HDLD1 or Tangier's disease is a recessive disorder characterized by absence of HDL cholesterol, accumulation of cholesteryl esters, premature coronary artery disease, hepatosplenomegaly, recurrent peripheral neuropathy and progressive muscle wasting and weakness (Brooks-Wilson *et al*, 1999; Bodzioch *et al*, 1999; Rust *et al*, 1999; Guo *et al*, 2002). HDLD2 or familial hypoalphalipoproteinemia is inherited an autosomal dominant trait. It is characterized by moderately low HDL cholesterol, susceptibility of premature coronary artery

disease and a reduction in cellular cholesterol efflux (Marcil *et al*, 1999; Brooks-Wilson *et al*, 1999). Other mutations and polymorphisms have been reported for this transporter and some common variation in noncoding regions may significantly alter the severity of atherosclerosis (Zwarts *et al*, 2002).

3.3.2. ABCB family

The ABCB family comprises 11 members, four of them are full transporters and the others are half transporters. In fact, this is the only subfamily that contains both full and half transporters. Mutations in some transporters of this subfamily are associated with genetic diseases.

The human bile salt export pump (BSEP) is a 1321 amino acid membrane protein with approximately 160kDa (Byrne *et al*, 2002) and comprises twelve transmembrane-spanning domains determining the substrate specificity and two typical and highly conserved intracellular nucleotide-binding domains with Walker A and B motifs required for binding and hydrolysis of ATP (Meier and Stieger, 2002; Trauner and Boyer, 2003). This protein is expressed in the canalicular micovilli and subcanalicular vesicles of the hepatocytes and mediates the excretion of mainly monovalent conjugated bile acids, being also involved in the transport of certain drugs (Childs *et al*, 1998; Lecureur e*t al*, 2000). The affinity of BSEP is low for unconjugated bile acids but high for conjugated bile acids, such as taurochenodeoxycholate (TCDCA), taurocholate (TCA), taurodeoxycholate (TDCA) and glycocholate (GCA) (Stieger *et al*, 2007). BSEP protein transport activity is inhibited by a wide diversity of compounds such as glybenclamide and troglitazone by direct binding of these compounds to BSEP, estradiol-17β-glucuronide, cyclosporine A, rifampicin and bosentan by inhibition of BSEP transporter function (Byrne *et al*, 2002; Fattinger *et al*, 2001). Furthermore, taurolithocholate induces the internalization of membrane vesicles which is involved in drug-induced cholestasis (Crocenzi *et al*, 2003a, b)

The human *ABCB11* gene is located on chromosome 2 at 2q24 on the reverse strand (Childs *et al*, 1998). *ABCB11* contains 28 exons and spans about 116kb. The *ABCB11* gene expression is highly regulated by the heterodimer FXR/RXR, with bile acids acting as ligands for FXR resulting in a feedforward regulation of BSEP by its substrates (Wang *et al*, 1999). Potential PXR response elements in human *BSEP* promoters were identified indicating a potential direct regulation of *BSEP* expression by PXR (Koesters and Karpen, 2008). The *ABCB11* gene regulation by ligands and nuclear receptors is complex and points to this gene as a critical component of the hepatocytes response to a variety of disease states and xenobiotics (Koesters and Karpen, 2008).

Mutations in the *ABCB11* gene lead to progressive familial intrahepatic cholestasis type 2 (PFIC 2), which is characterized by severe jaundice, hepatomegaly, failure to thrive and pruritus, whereas liver histology shows portal inflammation and giant-cell hepatitis (Bezerra and Balistreri, 2000). This disease has a rapid progressive course and generally leads to cirrhosis and liver failure with a need for liver transplant within the first decade of life.

Besides this phenotype, some mutations found in *ABCB11* lead to reduced transport activity when analysed in vitro, as result of decreased protein expression, altered membrane targeting and increased degradation by the proteasome (Wang *et al*, 2002a, b; Hayashi *et al*, 2005; Kagawa *et al*, 2008), whereas other variants were functional but unstable due to defective glycosilation (Plass *et al*, 2004).
Interindividual variability for basal BSEP protein levels was identified, but no correlation between protein expression and cholestasis markers was found. However a specific SNP (1457C) in *ABCB11* gene tended to be associated with low BSEP protein levels (Meier *et al*, 2006).

3.3.3. ABCC family

The ABCC family comprises 12 full transporters with a wide diversity of functions. They transport anionic or neutral drugs conjugated to acidic ligands and have an important function in resistance to nucleoside analogs (Borst *et al*, 2000a).
The human multidrug resistance protein 2 (MRP) is a 1545 amino acids membrane protein with a molecular mass of 174kDa. MRP3 is a 1527 amino acids membrane protein with a molecular mass of 169kDa (Uchiumi *et al*, 1998). Alternatively spliced variants which encode different protein isoforms have been described, but not all variants have been fully characterized (Fromm *et al*, 1999). MRP6 is a 1503 amino acids membrane protein with a molecular mass of 165kDa and is probably composed of 17 membrane spanning helices grouped into three transmembrane domains (Le Saux *et al*, 2000).
MRP2 is expressed at the canalicular (apical) membrane of human hepatocytes and it is also found in other apical domains of prolonged cells, such as the epithelial cells of proximal tubule of kidney and in the intestine. MRP3 is mainly expressed in liver, but it is also expressed in colon, small intestine and prostate and at a lower level in the kidney (Kiuchi *et al*, 1998; Kool *et al*, 1999a). MRP6 is expressed in the liver and kidney and at very low levels in other tissues (Bergen *et al*, 2000).
MRPs mediate the ATP-dependent transport of organic anions. MRP2 transports metabolites and clinically important drugs (usually conjugated with glutathione) such as anticancer drugs and also some carcinogens such as 2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine (PhIP). MRP3 mediates the transport of organic anions, monovalent and sulfated bile acids and phospholipids. It acts as an inducible transporter in the biliary and intestinal excretion of organic anions and as an alternative route for the export of bile acids and glucuronides from cholestatic hepatocytes. MRP6 may participate directly in the active transport of drugs into subcellular organelles or influence drug distribution indirectly. It transports glutathione conjugates as leukotriene-C4 (LTC4) and N-ethylmaleimide S-glutathione (NEM-GS) (Ilias *et al*, 2002).
MRP2 is involved in multi-drug resistance. An allosteric cross-stimulation mechanism was proposed for MRP2, in which bile acids and glucuronide conjugates are co-transported (Bodo *et*

al, 2003).Compounds such as taurocholate, penicillin G and pantoprazole stimulate estradiol 17-β-D-glucuronide transport by MRP2, but they themselves aren't transported by MRP2, suggesting that the stimulation of transport may involve two distinct binding sites, one for drug transport and other for allosteric regulation (Zelcer *et al*, 2003). MRP3 is induced in patients with Dubin-Johnson syndrome and in primary biliary cirrhosis (Konig *et al*, 1999), indicating, in the second case, an alternative mechanism for the export of bile salts. The MRP6 activity is inhibited by organic acids, such as indomethacin, probenecid and benzbromarone (Zhou *et al*, 2008).

These three human ABCCs genes are located in different chromosomes; *ABCC2* is localized on chromosome 10 at 10q24 (Taniguchi *et al*, 1996) and *ABCC3* is localized on chromosome 17 at 17q22, both on the direct strand (Uchiumi *et al*, 1998). *ABCC6* is localized on chromosome 16 at 16p13.1 on the reverse strand (Kuss *et al*, 1998). *ABCC2* contains 32 exons and spans about 200kb (Toh *et al*, 1999), *ABCC3* contains 31 exons and spans about 57kb and *ABCC6* contains 31 exons and spans about 74kb (Kool *et al*, 1999).

ABCC2 gene expression can be regulated by PXR, CAR and FXR. These nuclear receptors can activate *ABCC2* gene expression by a response element (ER-8) in the proximal promoter (Tanaka *et al*, 1999; Kast *et al*, 2002). The expression of *ABCC3* is regulated by LRH-1, which mediates bile acid-mediated transcriptional increase of gene levels, through two response elements located in the bile acid-responsive region (Inokuchi, 2001). *ABCC6* was found to be upregulated by retinoids, which act as agonists of RXR receptor (Ratajewski *et al*, 2006).

Mutations in *ABCC2* gene have been identified in Dubin-Johnson syndrome (DJS), a rare autossomal recessive disorder characterized by conjugated hyperbilirubinemia, an increase in the urinary excretion of copropophyrin isomer I, deposition of melanin-like pigment in hepatocytes and prolonged retention of sulfobromophthalein (Zimniak, 1993). The mutations range from point mutations to small deletions, leading to rapid degradation of mRNA, impaired protein maturation or inappropriate trafficking. Considering the important role of *ABCC2* from the pharmacological point of view, it was hypothesized that variants in this gene may contribute significantly to the pharmacokinetics and response of anticancer drugs, but this relationship still needs to be established. There are no description of associations between disease and MRP3. However, MRP3 may be the major transporter involved in basolateral transport of organic anions in liver, because animals deficient in this transporter have defects in transporting morphine 3-glucuronide in the liver into the blood stream (Zelcer et al, 2005). Besides, *ABCC3* has several polymorphisms, which can be important in the transport activity of this protein (Kobayashi *et al*, 2008). Mutations in *ABCC6* cause pseudoxanthoma elasticum (PXE) which is characterized by calcification of elastic fibers in skin, arteries and retina that results in dermal lesions with associated laxity and loss of elasticity, arterial insufficiency and retinal hemorrhages leading to macular degeneration (Bergen *et al*, 2000; Le Saux *et al*, 2000; Ringpfeil *et al*, 2000; Chassaing *et al*, 2005).

3.3.4. ABCG family

Members of the ABCG or White family are half-transporters which are thought to form dimers to become functionally active. These transporters are characterized by a nucleotide-binding domain at the N-terminus followed by six transmembrane-spanning domains (Klein *et al*, 1999).
The human breast cancer resistance protein (BCRP) is a 665 amino acid membrane protein with a molecular mass of 72kDa. The human ABCG5 is a 651 amino acid membrane protein with a molecular mass of 72kDa, while ABCG8 is a 673 amino acids membrane protein with a molecular mass of 76kDa. The human ABCG8 shares 28% sequence identity with ABCG5, containing both proteins a N-terminal ATP-binding motifs (Walker A and Walker B motifs) and the ABC transporter signature motif (C motif), and is predicted to contain 6 transmembrane segments in the C terminus (Berge *et al*, 2000). BCRP is highly expressed on the placenta, but it is also found in liver, small intestine, colon, kidney, heart and hematopoietic stem cells, while ABCG5 and ABCG8 are expressed in a tissue-specific manner in liver, colon and intestine.
BCRP can transport large, hydrophobic positively or negatively charged molecules, such as mitoxantrone, topotecan, SN-38, flavopiridol, methotrexate and several dyes. Its overexpression is sufficient to confer resistance to a broad profile of anticancer agents. Synthetic tyrosine kinase inhibitors also serve as strong substrates or inhibitors of BCRP. Although the physiological role of BCRP is unknown, it may have a role in the pharmacokinetic and pharmacodynamic profiles of some xenobiotics, protecting the body against toxins by obstructing intestinal absorption and mediating hepatobiliary excretion of its substrates. ABCG5/8 promotes sterol export and cholesterol (Langheim *et al*, 2005), while ABCG5/8 functions as a half-transporter to limit intestinal absorption and promote biliary excretion of sterols. ABCG5 appears to play an indispensable role in the selective sterol excretion by the liver into bile (Small, 2003).
The human *ABCG2* gene is located on chromosome 4 at 4q22; *ABCG5* and *ABCG8* are located on chromosome 2 at 2p21, all on the reverse strand. *ABCG2* contains 16 exons and spans about 66kb. The promoter has a CCAAT box but no TATA box, a potential CpG island and putative binding sites for SP1, AP1 and AP2 (Bailey-Dell *et al*, 2001). The *ABCG2* gene produces two transcripts that differ at the 5'-end but encode the same protein (Allikmets *et al*, 1998). *ABCG5* and *ABCG8* contain 13 exons and the first gene spans about 26.5kb while the second about 41kb (Berge *et al*, 2000). *ABCG8* gene is arrayed in a head-to-head orientation with *ABCG5* separated by 374bp (Berge *et al*, 2000). Assays of reporter gene activity with truncation mutants in the *ABCG2* promoter suggested the presence of positive and negative regulatory elements (Bailey-Dell *et al*, 2001). Increased levels of cholesterol were reported to increase *ABCG5/8* levels through LXR/RXR receptors (Repa *et al*, 2002). Activation of LXR was associated with an increased biliary cholesterol secretion, decreased fractional cholesterol absorption and increased fecal neutral sterol excretion (Yu *et al*, 2003).
There are no reports of association between disease and defects in *ABCG2*. Some polymorphisms in this gene were described as ethnic dependent and related with variations in

protein expression (Jong *et al*, 2004) or with an alteration in ATPase activity (Mizuarai *et al*, 2004). The functional consequences of these polymorphisms can be related with oral bioavailability of some drugs and may have an important role in terms of its toxicity (Kondo *et al*, 2004; Mizuarai *et al*, 2004). Defects in *ABCG5/8* are a cause of sitosterolemia, which is a rare autossomal recessive disorder, characterized by increased intestinal absorption of all sterols including cholesterol, plant and shellfish sterols and decreased biliary excretion of dietary sterols into bile. *ABCG8* normally cooperate with *ABCG5* to limit intestinal absorption and to promote biliary excretion of sterols, and that mutated forms of these transporters predispose to sterol accumulation and atherosclerosis (Berge *et al*, 2000). Patients frequently develop tendon and tuberous xanthomas, accelerated atherosclerosis and premature coronary artery disease (Berge *et al*, 2000). *ABCG5* and *ABCG8* are functionally polymorphic.

4. Molecular regulation of cholesterol homeostasis

4.1. By nuclear receptors

The expression of drug transporters and drug metabolizing enzymes in different organs is important to predict the pharmacological and toxicological effects of drugs. Therefore, interindividual variation in drug response is related to variability in the level of expression of genes involved in the process of drug disposition. Nuclear receptors have emerged as key metabolic sensors, regulating the expression of several genes involved in drug metabolism (Figure 7).

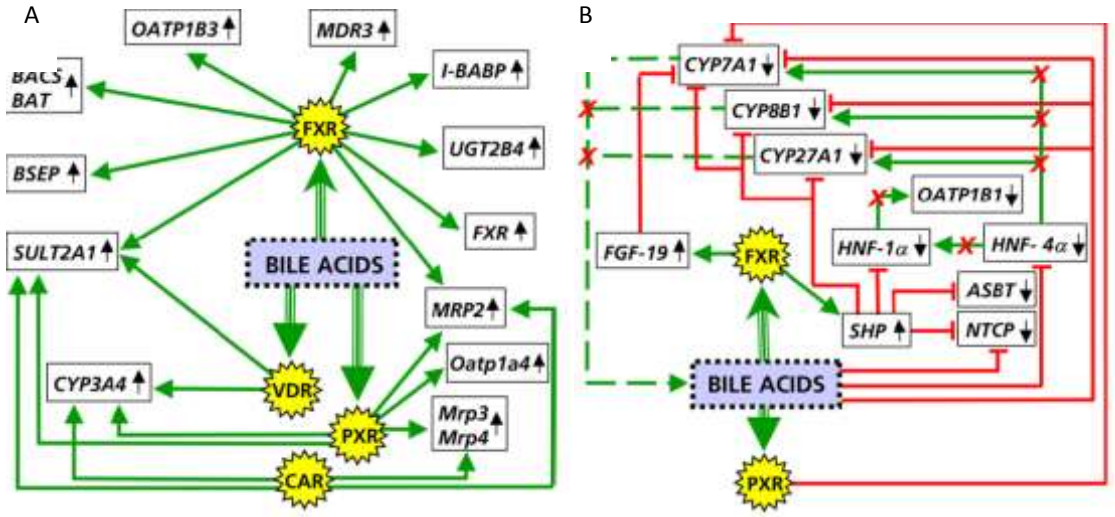

Fig.7 – Regulation of gene expression by bile acids via nuclear receptors. Genes regulated by bile acids that are directly or indirectly involved in bile acid metabolism are shown within square boxes in italics. The metabolic nuclear receptors involved in their regulation are shown within yellow stars. (A) Genes activated by bile acid-dependent mechanisms. Green lines indicate transcriptional activation of the indicated target genes. (B) Genes suppressed by bile acid-mediated mechanisms. Green and red lines indicate transcriptional activation and suppression, respectively, of the indicated target genes. The discontinuous green lines indicate bile acid synthesis. Red crosses over green lines indicate bile acid-mediated suppression of otherwise stimulatory pathways (Eloranta and Kullak-Ublick, 2005)

Nuclear receptors are ligand-activated transcription factors that can activate or inhibit gene expression. They are characterized by a central, highly conserved DNA-binding domain (DBD), a ligand-binding domain (LBD) in the C-terminus and a ligand-independent activation function 1 (AF-1) domain in the N-terminus of the nuclear receptor. The DBD specifically targets short recognition sequences of DNA in the promoters of genes, called response elements (RE), the LBD is responsible for ligand-binding and for dimerization and AF-1 coordinates the action of co-repressors and co-activators (Faber *et al*, 2003; Funk, 2008; Pelton *et al*, 2005).

Nuclear receptors in their inactive form are bound to co-repressors and after ligand binding their suffer a conformational change, which leads to dissociation of the co-repressor, association of co-activators and often dimerization with a partner, binding to a promoter site and activation of transcription of the target gene (Pelton *et al*, 2005).

Nuclear receptors are active as heterodimers, homodimers or a minority as monomers.

4.1.1. LXR

The liver X Receptor (LXR, NR1H) plays a critical role in the control of lipid metabolism, regulating gene transcription by binding as heterodimers with 9-cis retinoic acid receptor (RXR).

There are two LXR isoforms, LXRα (NR1H3) highly expressed in liver and LXRβ (NR1H2) ubiquitously expressed (Peet *et al*, 1999).
LXR ligands are the oxysterols, which are oxidized derivates of cholesterol and cholesterol precursor sterols, 24(S),25-epoxycholesterol being the most potent (Janowski *et al*, 1999).
LXR plays a key role in cholesterol homeostasis acting as cholesterol sensor and regulator of a set of genes involved in the absorption, transport, efflux and excretion of cholesterol. When LXR is activated, the LXR/RXR heterodimer induces the transcription of genes involved in cholesterol disposal from cells. *CYP7A1* is a target gene of LXR, leading to increased catabolism of cholesterol to bile acids. ABC transporters are also target genes of LXR, leading to increased removal out of the cell (Yu *et al*, 2003). However, synthetic LXR agonists lead to increased lipogenesis, increasing plasma triglyceride concentrations and hepatic steatosis (Fiévet and Staels, 2009).

4.1.2. FXR

The farnesoid X Receptor (FXR, NR1H) plays the role of master regulator of bile acid homeostasis, being the intracellular bile acid sensor. There are two FXR isoforms, FXRα, conserved from humans to fish and FXRβ, found in rodents, rabbits and dogs (Makishima *et al*, 1999). FXRα encodes four isoforms FXRα1, FXRα2, FXRα3 and FXRα4, resulting from different promoters and alternative splicing of the RNA (Lee *et al*, 2006b). FXR is highly expressed in liver, intestine, kidney and adrenals binding DNA on inverted repeated elements separated by one nucleotide (IR-1) (Modica *et al*, 2006).
FXR forms heterodimers with RXR upon its binding to bile acids and the FXR transcriptional modulation in the gut-liver axis synchronizes bile acid synthesis, conjugation, secretion, detoxification, uptake and excretion, thus modulating bile acid fate upon metabolic needs (Modica *at al*, 2006). *CYP7A1* and *CYP8B1* are target genes of FXR, leading to regulation of bile acid synthesis (Lee *et al*, 2006a). As previously mentioned other genes involved in bile acids uptake and secretion, such as some influx and ABC transporters are also regulated by FXR (Lee *et al*, 2006a; Lee at al, 2006b).

4.1.3. PPAR

The peroxisome Proliferator Activated Receptor (PPAR, NR1C) controls the expression the several genes involved in lipid and glucose metabolism. There are three PPAR isofoms, PPARα (NR1C1), PPARδ/β (NR1C2) and PPARγ (NR1C3) (Kota *et al*, 2005). PPAR is ubiquitously expressed throughout the body binding to DNA as a heterodimer with RXR, to direct repeat elements separated by one nucleotide (DR-1) (Juge-Aubry *et al*, 1997).

PPAR is activated by polyunsaturated fatty acids, eicosanoids and a variety of synthetic ligands, such as lipid-lowering fibrates (Chinetti-Gbaguidi *et al*, 2005).
PPAR plays a central role in the control of fatty acids and lipoprotein metabolism and in glucose homeostasis. Activation of PPAR induces the expression of genes controlling adipocyte fatty acid metabolism, but PPAR can also repress gene transcription either in a DNA-binding independent or dependent way, this mechanism being responsible for the anti-inflammatory activity of PPAR observed at the vascular and hepatic levels. In the DNA-binding independent manner, PPAR interferes with other signaling pathways or down-regulates membrane receptor expression; in the DNA-binding dependent manner PPAR recruits corepressors to unliganded PPAR (Chinetti-Gbaguidi *et al*, 2005).

4.1.4. LRH-1

The liver Receptor Homolog 1 (LRH-1, NR5A2) is important for the expression of the rate-limiting enzyme in bile acid biosynthesis, CYP7A1. LRH-1 is expressed mainly in liver, intestine, exocrine pancreas and ovary (Fayard *et al*, 2004). LRH-1 acts constitutively as a functional monomer in the absence of ligand (Tirona and Kim, 2005).
LRH-1 main target genes are *CYP7A1, α1-fetoprotein, ABCC3,* small heterodimer partner *(SHP),* cholesteryl ester transfer protein *(CTEP), ABCG5/ABCG8* among others (Francis *et al*, 2003).
LRH-1 has an important role in embryonic development and is also a major player in the regulation of cholesterol metabolism (Francis *et al*, 2003).

4.1.5. ROR

The retinoid-related Orphan Receptor (ROR, NR1F)) seems to play a major role in xeno- and endobiotic gene regulation. There are three ROR isoforms, RORα (NR1F1) expressed ubiquitously, RORβ (NR1F2) expressed in several regions of the central nervous system and RORγ (NR1F3) expressed in thymus, liver, kidney and muscle (Jetten *et al*, 2001).
ROR seems to be activated by cholesterol and its sulfonated derivatives, by melatonin and thiazolidinediones, although none of these compounds have been established as functional ligands (Wada *et al*, 2008).
ROR binds as a monomer to promoter regions in the target genes in a 6pb A/T-rich region immediately preceding a consensus AGGTCA motif and its transcriptional activity is negatively and positively regulated through the recruitment of other nuclear receptors (Wada et al, 2008).
ROR has an important role in tissue development, immune responses and circadian rhythm and can positively or negatively regulate the expression of drug metabolizing enzymes in a gene specific manner (Wada *et al*, 2008).

4.1.6. HNF4

Hepatocyte Nuclear Factor (HNF4) is a member of the orphan nuclear receptor superfamily and it is involved in the regulation of many genes that are preferentially expressed in the liver (Inoue *et al*, 2006). However HNF4α is also expressed in small intestine, kidney and pancreas.
HNF4α binds as a homodimer and is constitutively active upon binding of integral fatty acids (Wisely *et al*, 2002).
HNF4α plays a critical role in the regulation of drug disposition gene expression by regulating the constitutive expression of CYP genes, solute carriers and ABC transporters, as well as, other nuclear receptors (Li *et al*, 2000; Hayhurst *et al*, 2001; Jover *et al*, 2001).Among several functions, HNF4α plays an important role in liver development and differentiation and in lipid and bile acid metabolism (Hayhurst *et al*, 2001).

4.1.7. SHP

Small Heterodimer Partner-1 (SHP-1) is an unusual nuclear receptor without a DNA binding domain (Seol *et al*, 1996). It is expressed in liver, spleen and small intestine (Tirona and Kim, 2005) and it is activated by bile acid-mediated activation of FXR (Goodwin *et al*, 2000; Lu *et al*, 2000).
It heterodimerizes with other nuclear receptors to repress the expression of genes involved in bile acid synthesis, conjugation and transport (Tirona and Kim, 2005; Chiang, 2009). As an example, SHP-1 seems to have a major role in the down regulation of lipogenesis and in the protection against steatosis (Chiang, 2009).

4.1.8. PXR

The pregnane X receptor (PXR) is a promiscuous nuclear receptor expressed in liver, small intestine, colon and lymphocytes (Tirona and Kim, 2005). It is activated by a wide range of structural diverse exogenous chemicals such as rifampicin, pregnenolone 16α-carbinitrile (PCN) and taxol among others (Tirona and Kim, 2005) and also by endogenous compounds such as bile acids.
The induction of the target genes is initiated by ligand binding to PXR and posterior cytoplasmic-nuclear translocation. The ability of PXR to bind different compounds is facilitated by the ligand-binding pocket of this nuclear receptor that is smooth and large.
PXR is a master regulator involved in the coordinate induction of phase I and II drug metabolizing enzymes, as well as drug transporters that accelerate systemic clearance upon continued drug exposure (Tirona and Kim, 2005).

4.1.9. CAR

The constitutive Androstane Receptor (CAR) is a nuclear receptor expressed mainly in hepatocytes and enterocytes (Kawamoto *et al*, 1999). It is activated directly or indirectly by xenobiotics, such as phenobarbital, and by endogenous compounds, such as bilirubin, bile acids and steroids (Kawamoto *et al*, 1999).

After activation, CAR, that is present in the cytoplasm, translocates to the nucleus where it regulates the expression of genes encoding for drug metabolizing enzymes and influx and efflux transporters (Moore *et al*, 2000; Kim *et al*, 2001; Xiong *et al*, 2002). CAR regulates an overlapping set of genes with PXR and the mechanism of this cross-regulation has been attributed to the presence of shared responsive elements for these two nuclear receptors on the promoter region of the target genes (Wei *et al*, 2002).

CAR has a major role in the regulation of bilirubin clearance and bile acid detoxification. It is an unique nuclear receptor, that can respond to cytoplasmic signals that affect its nuclear translocation and it has a ligand-independent constitutive activity when localized in nucleus (Xie *et al*, 2003; Saini *et al*, 2004).

4.2. By other transcription factors

4.2.1. SREBP

Lipid homeostasis also can be regulated by transcription factors not belonging to the nuclear receptor superfamily, as the sterol regulatory element-binding proteins (SREBP). The SREBP family comprises three isoforms: SREBP-1a and SREBP-1c, which are derived from a single gene by alternative promoters and splicing and SREBP-2. All these isoforms are expressed in liver (Misawa *et al*, 2003).

SREBPs are integral membrane proteins of the endoplasmatic reticulum and the nuclear envelope and contain basic helix-loop-helix-zip (bHLH) domains (Brown and Goldstein, 1997).

When cells are depleted from sterols, proteolytically cleavage of SREBPs occurs by a two-step process and bHLH domain migrates to nucleus where it activates transcription of sterol response element (SRE)-containing genes (Sakai *et al*, 1998). Cleavage of the SREBPs is controlled by SREBP cleavage-activating protein (SCAP), which is regulated by the levels of sterols (Brown *et al*, 2002).

SREBPs are involved in the regulation of several genes involved in lipid metabolism, however SREBP-1a and SREBP-1c are responsible primarily for the regulation of genes involved in fatty acid biosynthesis, such as fatty acid synthase while SREBP-2 is primarily responsible for the

regulation of genes involved in cholesterol biosynthesis and metabolism, such as HMG-CoA reductase (Horton *et al*, 1998; Horton *et al*, 2002)

5. Cholesterol trafficking across intestine and liver

Cholesterol plays a central role in mammalian cells, but cellular demands as well dietary intake are widely variable, for example during development stages and in different nutritional states. This means that the body needs to have a constant circulation of cholesterol providing supply to all cells.

The liver has a crucial importance in the distribution of cholesterol to distinct pathways, such as the flux from the intestine to the liver; hepatobiliary excretion; the flux from liver to the periphery and the reverse pathway. Therefore, cholesterol flows within hepatocytes, enterocytes and peripheral cells by different transport routes in between various organs (Figure 8).

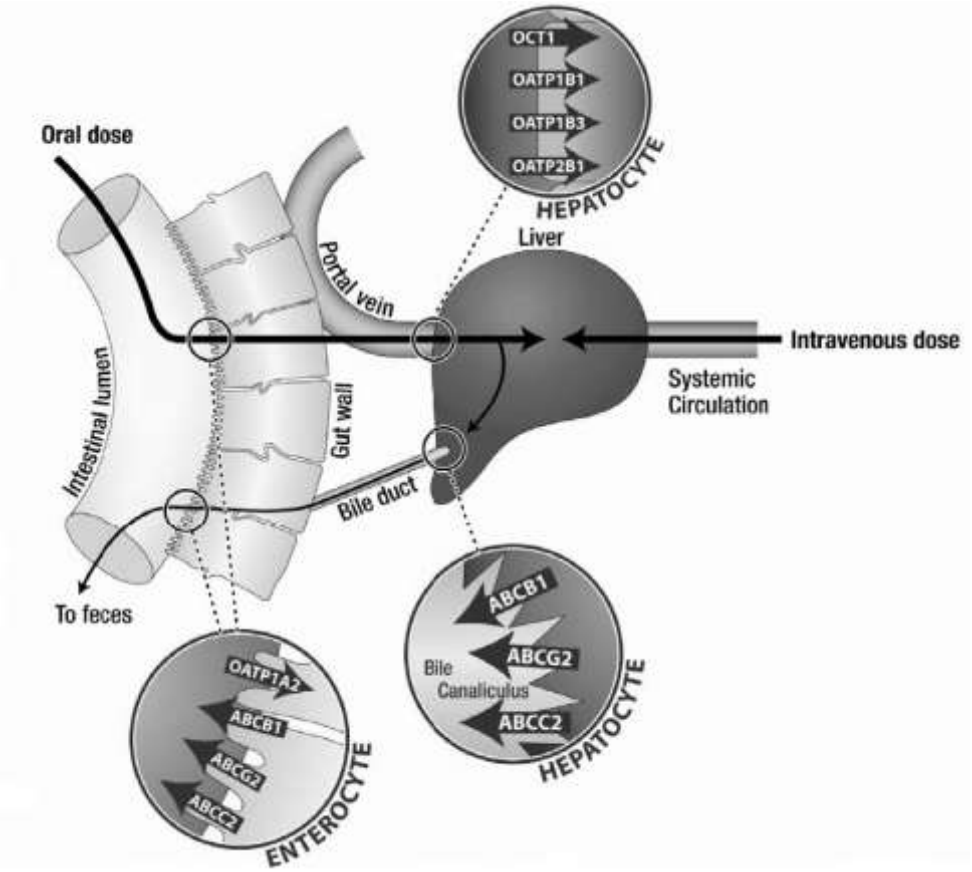

Fig.8 – Scheme showing the localization of ABC and SLC transporters involved in hepatic uptake and elimination in the bile (Franke *et al*, 2010)

5.1. Cholesterol flux in the enterocyte

Cholesterol from diet, bile and intestinal epithelial sloughing enters the small intestine where is absorbed by the enterocytes. Although cholesterol can be absorbed from lumen along all the small intestine, the major sites of absorption are in the duodenum and proximal jejunum.

The Niemann-Pick C1-like 1 (NPC1L1) protein is an influx transporter, localized at the apical membrane of the enterocyte and has a crucial importance for cholesterol absorption, allowing the passage of sterol across the brush border membrane of the enterocyte (Altman *et al*, 2004; Davis *et al*, 2004). After uptake by the enterocyte, acyl-CoA:cholesterol acyltransferase isoform 2 (ACAT2) esterifies cholesterol at C3 with fatty acids to form cholesteryl ester. Plant sterols, however, remains unsterified, this being the main difference between plant sterols and cholesterol for all reactions in enterocyte. Cholesteryl esters are subsequently packaged into chylomicra and secreted to the lymph, ultimately reaching the liver (van Greevenbroek *et al*, 1998). Unsterified plant sterols and some cholesterol are excreted back to the intestinal lumen by ABCG5 and ABCG8, LXRα being essential for the upregulation of these efflux transporters in response to high dietary cholesterol.

As intestinal absorption is a multistep mechanism that is regulated by several genes in the enterocyte, the combined regulatory effects of NPC1L1, ABCG5 and ABCG8 may play a major role in modulating the amount of cholesterol that reaches the lymph from the intestinal lumen (Wang, 2007).

5.2. Cholesterol flux in the hepatocyte

The liver is the main organ for cholesterol homeostasis, being the point of intersection of various metabolic pathways. A considerable amount of cholesterol synthesis occurs in the liver and this cholesterol has to be distributed to the target organs.

The hepatocyte has another important role in cholesterol metabolism since it can excrete cholesterol from the body via bile acids. Furthermore, hepatocytes are also the major suppliers of lipoproteins, which have an important role for redistribution of cholesterol around the body.

Chylomicron remnants, intermediate density lipoprotein (IDL) and LDL are taken up by receptor-mediated endocytosis in hepatocytes and the scavenger receptor SR-BI is responsible for the selective uptake of cholesterol from HDL without internalization of the complete particle (Maxfield and Wüstner, 2002). Inside the hepatocyte cholesterol is stored as cholesteryl esters, which can be hydrolyzed to free cholesterol and released into the cell, where it can be shuttled into bile, re-esterified and used for assembly of very low density lipoproteins (VLDL) or be converted to bile salts and undergo hepatobiliary secretion (Kang and Davis, 2000). Assembled VLDL is secreted at the basolateral membrane of hepatocytes and cholesterol and cholesteryl

esters can be excreted to systemic circulation. These molecules can also enter the systemic circulation via HDL pathway.

5.3. Enterohepatic circulation

The cycling movement of bile acids from the liver to small intestine and back to liver is referred as enterohepatic circulation. Bile acids are synthesized in hepatocytes from cholesterol and they are conjugated with taurine or glycine to bile salts and can be stored in gallbladder. Bile salt secretion is the major force for bile flow.

Bile salts are excreted from the hepatocyte to canalicular bile through BSEP, while water passively follows the osmotic gradient originating the bile flow (Chiang, 2009). Phospholipids are translocated by ABCC2/ABCC3 and ABCG5/ABCG8 are responsible for the cholesterol transport to bile (Chiang, 2009).

In the intestinal tract, a small part of bile salts and cholesterol may undergo bacterial transformation and be excreted in the feces, while a substantial part is taken up in the intestine and re-enters in the body. The bile salts are reabsorbed in the ileum by the apical sodium-dependent bile acid transporter (ASBT) located in the brush border membrane. Bile salts are trans-diffused across the enterocyte to the basolateral membrane. Here the organic solute transporter (OST) effluxes bile acids into portal blood circulation, where they are transported to the basolateral membrane and return into hepatocytes by NTCP (Hofmann, 2009).

5.4. Reverse transport of cholesterol

The transfer of cholesterol from nonhepatic cells to liver is referred as reverse cholesterol transport.

Cholesterol can be carried through circulation as a component of several lipoproteins, of which LDL is responsible for the transport into peripheral cells and HDL has the opposite function, removing cholesterol from peripheral cells and transporting it back to liver (Figure 9). Therefore, it is considered that this reverse cholesterol transport is a positive characteristic of HDL, being beneficial against the development of atherosclerosis. Furthermore, HDL has been described to have anti- inflammatory and antioxidant properties, which contribute to its beneficial profile (Kontush and Chapman, 2006).

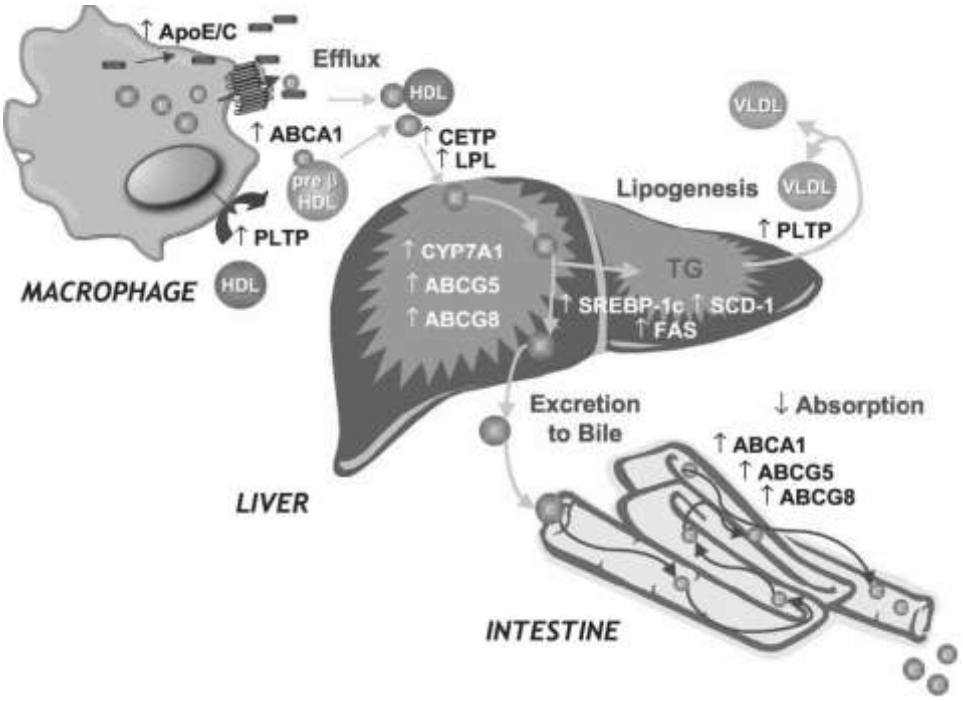

Fig.9 – Cholesterol Transport (Tontonoz and Mangelsdorf, 2003)

ABCA1 transfer cholesterol and phospholipids to lipid-poor pre-β-HDL particles, forming HDL, which besides this lipid content have a protein component, ApoA-1 (Greenfeder, 2009). Cholesterol in nascent HDL is esterified by lecithin-cholesterol acyltransferase (LCAT), originating mature HDL. Cholesteryl ester transfer protein (CETP) mediates the exchange of cholesteryl esters from HDL to triglyceride-rich particles and HDL receives triglycerides, which are subsequently hydrolyzed. This concerted action of CETP helps to regenerate cholesterol-poor particles, which are the preferred cholesterol acceptors (Greenfeder, 2009). Mature HDL also can bind to SR-BI at the membrane of the target cell mediating the selective uptake of cholesterol and cholesteryl esters to the accepting plasma membrane (Assmann *et al*, 2004).

6. Pathological situations associated to cholesterol

Although cholesterol is an essential component for human life, high levels in circulation are associated to pathological situations. Therefore all processes involved in maintenance of cholesterol homeostasis are tightly regulated.

The most common pathological situation associated to high levels of cholesterol is atherosclerosis, which is a prerequisite for the majority of cases of coronary heart disease, the first cause of death in developed countries.

Cholesterol carried by lipoproteins in blood circulation can accumulate in macrophages in the vessel walls, leading to development of atherosclerotic plaques and subsequently impair the

blood flow and in advanced stages stop blood flow, leading to death of the tissues fed by the artery, for example heart or brain (Puddu *et al*, 2005).

Coronary heart disease seems to be correlated with plasma cholesterol levels and circulating liproproteins (Breslow, 2000). Therefore high levels of LDL are associated with the development of atherosclerotic plaques, leading to the term of bad cholesterol, while HDL, referred as good cholesterol, is correlated to a protective effect (Abrahamsson *et al*, 2005; Assmann *et al*, 2004; Greenfeder, 2009). These correlations rise from the direction of cholesterol circulation, LDL transports cholesterol from the liver to peripheral cells and HDL is involved in the reverse transport of cholesterol.

7. Genetic variability and response to treatment

In the last decades it has been observed that patients respond differently to the same treatment, highlighting the importance of pharmacogenetics. Genes involved in the transport and in the metabolism of drugs are highly polymorphic and some of these genetic variations are already associated with altered response to treatment. The knowledge on pharmacogenetic markers for cardiovascular diseases as well as on the molecular mechanisms of regulation of genes involved in disease progression can be extremely important, not only to obtain adequate knowledge on drug pharmacokinetics but also to predict occurrence of potential adverse drug interactions. This kind of studies has an extreme importance since it opens the door to a new way of treatment, the personalized therapy. Knowing the genetic background of a population allows developing new, more effective and safe therapeutics for each person. An evaluation of SNP frequencies among different populations with variable ethnic background will certainly be useful to characterize the changes in drug pharmacokinetics and predict differential susceptibility to possible adverse effects.

The cholesterol lowering drugs in use are mainly HMG-CoA reductase inhibitors (statins), fibrates, ezetimibe and bile-acid binding resins that act reducing the levels of cholesterol by different pathways (Schmitz and Lagmann, 2006). Clinically relevant drugs are transported and metabolized by polymorphic proteins. Some of these SNPs are associated to altered function of the protein (Schmitz and Langman, 2005; Schmitz *et al*, 2001), which influence drug pharmacokinetics and also can cause variable drug disposition, and as consequence treatment efficacy.

Chapter II - Aims

Aims

Little is known on the variability in the genes coding for proteins that are involved in the control of lipid homeostasis, mediating biotransformation and transport. On the other hand, there is very limited information on how changes in the levels of endogenous lipids may affect expression of these genes. Since some of these enzymes and transporters are also able to act on therapeutic drugs, it is conceivable that therapeutic outcome may differ among individuals due not only to pharmacogenetic variability but also due to differential expression of these genes depending on physiological factors, or to the presence of pathology.

The overall aim of the present study was to characterize selected genes coding for drug metabolizing enzymes and influx and efflux transporters both from the point of view of pharmacogenetic diversity and from their response to increased cholesterol concentrations.

The specific objectives were:

1) To characterize, in a sample of the Portuguese population, selected genetic polymorphisms in cytochromes P450, influx and efflux transporters that are known to – or that may be expected to - affect *in vitro* or *in vivo* the metabolism or transport of cholesterol and/or therapeutic drugs

2) To evaluate the existence of ethnic variability in the frequency of these polymorphic variants, through the determination of their prevalence in other populations

3) To investigate to which extent the expression of these genes, in liver and intestine derived cells, may be influenced by the accumulation of cholesterol.

Chapter III - Results

1. Pharmacogenetics of genes candidate to play a role in cardiovascular disease and therapeutics

Paper I – Polymorphisms in SLCO1B1, SLCO1B3, SLC22A1 and SLC10A1 transporters among Caucasians, native Africans and South Americans – (Submitted to European Journal of Clinical Pharmacology)

Paper II – Ethnic differences in the prevalence of polymorphisms in drug metabolizing enzymes involved in cholesterol metabolism – (Manuscript)

Paper III – Inter-ethnic variability in ABC transporters ABCB11, ABCG5 AND ABCG8 – (Manuscript)

POLYMORPHISMS IN *SLCO1B1*, *SLCO1B3*, *SLC22A1* and *SLC10A1* TRANSPORTERS AMONG CAUCASIANS, NATIVE AFRICANS AND SOUTH AMERICANS

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ABSTRACT

Purpose: It is well known that drug disposition and response are greatly determined by the activities of drug metabolizing enzymes and transporters. Uptake transporters have recently emerged as key contributors to the process of drug disposition. Polymorphisms of uptake transporters have been demonstrated to influence *in vitro* transport function and the pharmacokinetic profile of compounds. Therefore, the pharmacogenetic analysis of influx transporters might help to personalize and optimize drug therapy. Due to ethnic variability in polymorphism distribution, some populations may be at higher risk for adverse events, treatment failure or prevalence of pathologies.

Methods: In this study, novel PCR-RFLP methods were developed to investigate the presence of thirteen polymorphic variants in the *SLCO1B1* (OATP-C), *SLCO1B3* (OATP-8), *SLC22A1* (OCT1) and *SLC10A1* (NTCP) genes in subjects from Portugal, Mozambique and Colombia.

Results: The variants C800T in *SLC10A1* and T217C in *SLCO1B1* were not detected in any population studied. High frequencies were determined for T4215C and C83G (*SLC22A1*), A2192G (*SLC10A1*) and G699A (*SLCO1B3*), with a similar distribution in individuals from the three populations. G1564T (*SLCO1B3*) was present in a low frequency in the three different ethnic groups. Significant differences were observed for A2587G (*SLC10A1*), C480G (*SLC22A1*), A388G, C463A and T521C (*SLCO1B1*) and T334G (*SLCO1B3*).

Conclusions: Our results demonstrate a high variability in the prevalence of influx transporters' variants among the three populations analyzed, contributing information about these potentially relevant SNPs in Africa and South America, regions for which data on pharmacogenetic variation is dramatically lacking.

Keywords: uptake transporter, ethnic, pharmacogenetics, SLCO1B1, SLCO1B3, SLC10A1, SLC22A1

INTRODUCTION

Cardiovascular diseases are the major causes of death and morbidity in developed countries. The management of cardiovascular diseases is critically dependent on lipid-lowering therapy [1]. The response to these drugs is highly variable and pharmacogenetic diversity may in part contribute to this phenomenon.

Organic anion transporting polypeptides (OATPs) are basolateral plasma membrane transport proteins that mediate the sodium-independent influx of a wide range of amphipatic organic compounds including bile salts, steroid conjugates, thyroid hormones, anionic oligopeptides and conjugated and unconjugated bilirubin [2]. In addition to this physiological function, OATPs have been found to transport an increasing number of frequently used drugs, such as several HMGCoA reductase inhibitors, fexofenadine, benzylpenicillin, repaglinide, valsartan, and temocaprilat [3, 4]. Within this family of transporters, OATP-C (SLCO1B1/OATP1B1) and OATP-8 (SLCO1B3/OATP1B3) are important drug and hormone uptake transporters in liver since they are located in the sinusoidal membrane of the hepatocyte, the main gate to the entrance of xenobiotics and endogenous substrates. These two transporters display several polymorphisms which may contribute to interindividual variability in drug disposition and response [5, 6]. Indeed, polymorphisms in *SLCO1B1* and *SLCO1B3* have recently been associated with altered pharmacokinetics of pravastatin, pitavastatin, simvastatin, atorvastatin, rosuvastatin, repaglinide, nateglinide, fexofenadine, atrasentan, paclitaxel, digoxin and also with variable changes in cholesterol levels following statin administration [3, 5-14]. Considerable differences have been reported in the prevalence of polymorphisms in *SLCO1B1* and *SLCO1B3* among populations from different ethnic origins [15, 16].

NTCP (SLC10A1) is the basolateral (sinusoidal) Na^+-bile acid co-transporter that plays a central role in the hepatic uptake of bile acids, being also responsible for the transport of drugs, including statins [17]. Changes in the expression or function of SLC10A1 would be predicted to significantly affect enterohepatic circulation of bile acids and directly affect cellular signalling pathways importantly involved in cholesterol homeostasis and hepatocyte function. In fact, NTCP was described as being induced in response to cholesterol [18]. Functional polymorphisms are known to exist among bile acid transporter family members but little is known regarding genetic heterogeneity in NTCP [19].

The organic cation transporter 1 (OCT1, SLC22A1) is another transporter protein that mediates the uptake of a variety of endogenous compounds (eg. noradrenaline, acetylcholine) and cationic drugs, such as cimetidine, clonidine, procainamide, desipramine, protease inhibitors, phenformin, midazolam and acyclovir in an electrochemical potential-dependent manner [20]. Since SLC22A1 is primarily expressed in the liver, it is expected to play a fundamental role in the uptake of substrates into the liver. This was confirmed in SLC22A1-knockout mice, which exhibited decreased liver accumulation of the anti-tumor drug metaiodobenzyl-guanidine and the antidiabetic drug metformin [21]. As NTCP, OCT1 was described as being induced in response to cholesterol [18] and several genetic polymorphisms have been found in *SLC22A1* in different ethnic groups [22].

Considering that these transporters play a key role in the distribution of many drugs and in the transport of endogenous compounds, such as cholesterol and bile acids, inter-individual variability in disease risk and drug response may be explained by the differential prevalence of genetic variants. In the present study we examined the allelic frequencies of thirteen SNPs in *SLC10A1*, *SLC22A1*, *SLCO1B1* and *SLCO1B3*, that may play an important role in drug disposition, in populations from three different ethnic/geographic origins. Native Africans and Latin American populations are not frequently the target of pharmacogenetic studies, and this is an important issue when considering bridging of drug dosages and regimens used in Caucasians.

METHODS

Subjects. The populations under study consisted of 92 Portuguese, 151 Mozambican and 91 Colombian subjects. The populations from Mozambique and Portugal were described elsewhere [23, 24]. Colombian subjects were from the North-West region, mainly from Antioquia and Chocó Departments (62 men, 27 women). This study followed the recommendations of the Declaration of Helsinki, promulgated in 1964 (http://ohsr.od.nih.gov/helsinki.php3). Genomic DNA was extracted from whole peripheral blood, using the Qiamp DNA Extraction Kit (Qiagen) or Chelex (BioRad) according to the manufacturer's protocols.

Pharmacogenetic analysis of polymorphic variants. Each of the thirteen SNPs analysed in *SLC10A1*, *SLC22A1*, *SLCO1B1* and *SLCO1B3* (reference sequences, GenBank accession no. NC_000014.7, NC_000012.10, NC_000012.10, NC_000012.10, respectively) were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays.

Table 1 lists the primers employed in performing PCR for the analysed SNPs.

Table 1: Primers used in PCR amplification

Gene	Polymorphism	Forward primer (5'→3')	Reverse Primer (5'→3')
SLC10A1	C800T	ACTTGCTTGTTGGCAGGCTCAGGTC	AGATTCCATCTGCTGCGAAACTC
	A2587G	ACTCTGGGAAACCTCTGGCAAGTC	TCTGGGTGGCTCCGGAACCTG
	A2192G	AGCCACCCAGACCTGGAGCCAATGT	ATAAGCTCTGCTGGGACAGCCACC
SLC22A1	C480G	ACTGGCAGTTCAACCTGGTGTGTGC	TCATGACATTCAGAGGAGGCTTACC
	C83G	TGGCAATCCCACTGCAATGGCTACC	AGACTCTGCGAAGCACTCAC
	T4215C	AGGGCTGCCTAGTGTGTGAGGTC	TCAGCCATACAACATCCAGG
SLCO1B1	T217C	ATTGCTAAGACACTAGGTGC	ATTGCCAAATTGCCTGTGAG
	A388G C463A	GCAAATAAAGGGGAATATTTCTC	AGAGATGTAATTAAATGTATAC
	T521C	GTTAAATTTGTAATAGAAATGC	GTAGACAAAGGGAAAGTGATCATA
SLCO1B3	T334G	AAGGTACAATGTCTTGGGCA	TCCTCTCAAAAGGTAACTGC
	G699A	AGTCATTGGCTTTGCACTGGGATC	TCTTTGGAAGAATGGTGTCC
	G1564T	TGTGTGGAAGTAACTGGTCTCCAGA	TGGTACCTCCTGTTGCAGAGA

Genomic DNA was added to PCR mixtures of 25µl consisting of 1× PCR buffer, 1-4mM $MgCl_2$ (Promega), 0.2mM dNTPs (Promega), and 1.5U of Go *Taq* Polymerase (Promega). PCR amplifications were conducted (Tpersonal Themocycler, Biometra) using one denaturation step at 94ºC for 2min, followed by 30-35 cycles of 94ºC for 30s, 40-55ºC for 30s and 72ºC for 1min and a final PCR extension of 3min at 72ºC.

Genotype assignment was performed by digestion of amplification products with specific restriction endonucleases (Table 2), separation by electrophoresis on 8% or 10% (W/V) poliacrylamide gels and visualized with ethidium bromide staining under UV light (AlphaImager, AlphaInnotech).

Table 2: Restriction Endonucleases used in PCR-RFLP

Gene	SNP	Aminoacid change	Fragment length of PCR product (bp)	Restriction Enzyme	Cleavage pattern (bp)
SLC10A1	C800T	S267F	307	HphI	CC: 160+147 TT: 307
	A2587G	-	380	Hpy8I	AA: 214+166 GG: 380
	A2192G	-	355	FaqI	AA: 176+107+72 GG: 283+72
SLC22A1	C480G	F160L	156	MboII	CC: 91+65 GG: 156
	C83G	-	385	BtsI	GG: 356+29 CC: 385
	T4215C	-	210	NdeII	TT: 124+86 CC: 210
SLCO1B1	T217C	L73F	226	HindIII	TT: 226 CC: 127+99
	A388G	N130D	274	ClaI	AA: 274 GG: 155+119
	C463A	P155T	274	HphI	AA: 274 CC: 185+89
	T521C	V174A	260	Bsp1286I	TT: 260 CC: 153+107
SLCO1B3	T334G	S112A	344	AluI	TT: 252+71+21 GG: 212+71+40+21
	G699A	M233I	135	RsaI	GG: 74+45+16 AA: 90+45
	G1564T	G522C	145	HphI	TT: 145 GG: 86+59

Statistical analysis. Fisher's exact test was used to assess Hardy-Weinberg equilibrium. Statistical analyses were performed using the Student's *t*-test and the χ^2 test.

RESULTS

A total of thirteen *SLC10A1*, *SLC22A1*, *SLCO1B1* and *SLCO1B3* genetic variants were genotyped in a sample of 92 Portuguese, 151 Mozambican and 91 Colombian subjects. The frequencies for allelic variants of *SLC10A1*, *SLC22A1*, *SLCO1B1* and *SLCO1B3* in the three population samples are shown in table 3.

Table 3: *SLC10A1*, *SLC22A1*, *SLCO1B1* and *SLCO1B3* variant allelic frequencies (%) in subjects of different ethnic groups

Gene	SNP (aa change)	dbSNP ID	Portugal (n=184)	95% CI	Mozambique (n=302)	95% CI	Colombia (n=182)	95% CI
SLC 10A1	C800T (S267F)	rs2296651	0	0.00-4.81	0	0.00- 2.98	0	0.00- 4.81
	A2587G (-)	rs943276	70.7	66.83-83.71	98	77.59-102.65**	76.4	73.50-8.119
	A2192G (-)	rs943277	34.8	28.78-48.27	24.2	10.79-25.48	28	21.94-40.50
SLC 22A1	C480G (F160L)	rs683369	22.3	16.29-33.62	1.3	0.00-2.98**	20.3	14.46-31.28
	C83G (-)	rs3798172	16.3	10.89-26.50	17.5	7.54-18.02	17	11.77-27.71
	T4215C (-)	rs654993	31.5	25.82-44.97	20.2	18.81-29.52	30.2	23.87-42.74
SLCO 1B1	T217C (F73L)	rs56101265	0	0.00-4.81	0	0.00- 2.98	0	0.00- 4.81
	A388G (N130D)	rs2306283	42.4	35.85-55.80	65.6	56.80-71.37*	47.8	42.09-62.09
	C463A (P155T)	rs11045819	23.4	17.22-34.78	16.6	7.01-17.27	5.5	2.75-13.78**
	T521C (V174A)	rs4149056	15.3	10.02-25.29	4	0.80-6.80*	14.8	10.02-25.29
SLCO 1B3	T334G (S112A)	rs4149117	70.1	66.83-83.71	22.2	9.69-23.01**	77.5	75.94-90.84
	G699A (M233I)	rs7311358	70.1	66.83-83.71	59.3	31.43-67.75	71.4	67.54-84.62
	G1564T (G522C)	rs4149056	5.4	2.04-12.40	3.3	0.41-5.90	4.4	1.36-11.00

** p<0.001
* p<0.05

For *SLC10A1* (NTCP), the variant 800T was not detected in any of the three populations studied, in contrast with A2587G that showed a high frequency for the three populations studied (70.7% for Portuguese, 76.4% for Colombian and 98% for Mozambican individuals), significantly higher for the population of Mozambique (p<0.001). Frequencies for the A2192G variant were similar among the three populations analysed.

For *SLC22A1* (OCT1), the 480G allele was more prevalent in Portuguese (22.3%) and Colombian (20.3%) samples compared with Mozambican individuals (1.3%, with $p<0.001$). The frequencies of 4215C and 83G alleles were similar between the three populations.

For *SLCO1B1* (OATP-C), the variant 217C was not detected among the individuals analysed from Portugal, Mozambique and Colombia. The variant 388G had a high frequency for the populations studied, 42.4% for Portuguese, 47.8% for Colombian with significant differences for Mozambican individuals (65.6%, with $p<0.05$). The variant 463A showed similar frequencies between Portuguese and Mozambican subjects (23.4% and 16.6%, respectively), while significant differences from Colombian subjects were determined (5.5%, with $p<0.01$). In the case of the polymorphism T521C similar frequencies were observed in Portuguese (10.3%) and Colombian (14.8%) while the Mozambican population differed significantly (4%, with $p<0.01$).

For *SLCO1B3* (OATP-8), the 699A allele was highly prevalent in all populations studied, in contrast with G1564T that was found to be rare in the three different population samples. The variant 334G was frequent in Portuguese (70.1%) and Colombian (77.5%) but less frequent in Mozambican (22.2%, with $p<0.01$) individuals.

None of the genetic variants showed any significant deviation from Hardy-Weinberg equilibrium.

DISCUSSION

Influx transporters play a key role in the disposition of many drugs such as antidislipidemic, anti-tumor or antidiabetic, and in some cases have been demonstrated to affect efficacy and/or safety. As the response to a large range of drugs is highly variable, pharmacogenetic diversity may in part contribute to this variability, being important in the context of therapeutic efficacy and safety. An evaluation of SNP frequencies among different populations with variable ethnic background will certainly be useful as a tool to optimize therapeutics according to variable predicted pharmacokinetics.

We analyzed the frequencies of thirteen SNPs in four uptake transporters in individuals from Portugal, Mozambique and Colombia. From these SNPs, *SLC10A1* C800T and *SLCO1B1* T217C were not detected in any of the three populations in study, which is in agreement with previous reports [15, 19]. *SLC10A1* C800T results in the amino acid exchange Ser267Phe and is predicted to be localized in the third putative extracellular loop, a region of the protein that could be considered a signature motif for NTCP transporters [19]. Furthermore, this variant seems to display different substrate selectivity, since this mutation was shown to lead to a near complete loss of bile acid uptake, but fully normal uptake activity for the non-bile acid substrate recognition [19]. *SLCO1B1* T217C results in the amino acid exchange Phe73Leu, an aminoacid highly conserved among the rat and mouse orthologs [25]. This change is localized in the putative transmembrane-spanning domain and was associated with a significant reduction in transport activity [25].

To the best of our knowledge, this is the first description of the intronic SNPs *SLC10A1* A2587G and A2192G. The allelic frequencies obtained in this study for these SNPs were very high, especially in the case of A2587G. Since polymorphisms in the exons of *SLC10A1* are relatively rare, suggesting evolutionary conservation in the transport of endogenous compounds such as cholesterol and bile acids, intronic SNPs may be important as a source of variability, since they could affect the mRNA splicing or give origin to premature STOP codons and to non coding transcripts.

The high allelic frequency for *SLC22A1* C480G (Phe160Leu) found in Portuguese is in agreement with a previous study in another Caucasian population [26]. However, this allelic frequency is higher than reported in a European American population (6.5%), a Mexican American population (5%) and an African American population (0.5%) [27]. This discrepancy may be caused by differences in sample sizes between these studies and in the origin of the samples. The high frequency determined in Colombians is consistent with the European (Spanish) ancestry of a wide percentage of the population. Phe160Leu is located in the middle of the second transmembrane domain [28] and, although no changes in substrate affinity and selectivity were reported for this mutation [26, 28], this SNP was recently associated with high rate of loss of response or treatment failure to imatinib mesylate therapy, compound used in the treatment of chronic myeloid leukaemia [29]. The differences observed in the frequencies between Caucasians/Colombians and Mozambican raise the question of a possible contribution to differential risk of therapeutic failure among these populations.

The allelic frequencies obtained for *OCT1* 83G and 4215C were high but similar between the three populations studied and to our knowledge this represents the first examination of intronic polymorphisms for this uptake transporter.

The allelic frequency obtained for *SLCO1B1* 388G (Asp130) in Portuguese is in agreement with the frequencies obtained by other authors in Finnish and German Caucasians [8, 15]. In the population of Mozambique the allelic frequency was similar compared with other studies in Africans from Uganda (77.8%) [15] and Afro-Americans (75%) [25]. This SNP localized in extracellular loop 2 seems to be associated only with minimal changes in the transport activity [25]. However, recently Pasanen *et al* reported that the SLCO1B1 transporter may be important in the regulation of cholesterol synthesis and that the variant 388G is associated with an increase of cholesterol synthesis [30].

The allelic frequency of *SLCO1B1* C463A (Thr155) for the Portuguese population was 23.4%, value that is slightly higher than the reported previously for Germans (16%) [15] and for Finish (13.1%)(Pasanen *et al*, 2006), but this difference is not statistically significant. Surprisingly, when comparing the allelic frequencies of *SLCO1B1* 463A in Mozambique with another African population from Uganda [15] we can see significant differences in the frequency of this SNP in those populations. This could be due to the different ethnic groups that exist in Mozambique (African 99.66% -Makhuwa, Tsonga, Lomwe, Sena, and others, Europeans 0.06% - Euro-Africans 0.2%, Indians 0.08%) and Uganda (Baganda 16.9%, Banyakole 9.5%, Basoga 8.4%, Bakiga 6.9%, Iteso 6.4%, Langi 6.1%, Acholi 4.7%, Bagisu 4.6%, Lugbara 4.2%, Bunyoro 2.7%, other 29.6%). This SNP is predicted to be localized on extracellular loop 5 and was associated with a significant reduction in transport of estrone sulphate and estradiol 17β-D-glucuronide, suggesting an important role of this region to substrate-transporter interaction [25].
The *SLCO1B1* 521C allele (Ala174), which has been associated with reduced SLCO1B1 activity [5, 25, 31] was found in an allelic frequency of 15.3% for Portuguese individuals, which is in agreement with previously reports for Germans (15%) [15] and Finnish (20.2%) [4]. The results for the Mozambicans (4%) are similar to the results reported to another African population (3.9%) [15]. The Colombian population had an allelic frequency of 14.8%, which is close to the result obtained to the Portuguese. It is becoming increasingly evident that this SNP has a major effect on SLCO1B1 activity, affecting the plasma concentrations of several drugs [5, 25, 31]. In fact, subjects carriers of both variants 388G and 521C (haplotype *SLCO1B1*15*) exhibited elevated systemic exposure to pravastatin as compared to subjects with wild type alleles [9, 31]. 521C is associated with pharmacokinetic alterations of several drugs besides some statins, such as repaglinide, an antidiabetic drug; fexofenadine, an antihistamine; atrasentan, an endothelin A receptor antagonist [13] and lopinavir, an antiretroviral [32, 33]. This polymorphism was also associated with an increased risk of cardiovascular adverse effects in individuals taking simvastatin [14].
SNPs described in *SLCO1B1* have been shown to be important in drug metabolism, since they influence pharmacokinetic parameters of several drugs in therapeutical use, such as some statins, including pravastatin [31], pitavastatin [34], simvastatin [8], torasemid [35], repaglinide

[5], fexofenadine [36] and atorvastatin [37]. Substrates of SLCO1B1 are known ligands for nuclear receptors such as the pregnane X receptor (PXR), which can explain the contribution of this transporter to the transcriptional regulation of other enzymes and transporters. Therefore, it has been suggested that subjects with SNPs in *SLCO1B1* may be less susceptible to drug modulation of this transporter and other proteins [38].

The allelic frequency of *SLCO1B3* 334G (Ala112) for the Portuguese population was 70.1% and for the Mozambican was 22.2%, values slightly reduced than that reported previously in European (74%) [6], European Caucasian (81%), American Caucasian (88%), Afro-American (41%) and Ghanaian (38%) [10]. The allelic frequency obtained for the Colombian (77.5%) was similar to the one reported to a Mexican population (78%) [10].

The allelic frequency of 699A (Ile233) for the Portuguese population was 70.1%, value similar to the reported for Europeans (71%) [6], and smaller than that reported in Americans (87%) [10]. For Mozambicans the allelic frequency in this study was 59.3%, higher than the reported to Afro-Americans (41%), while the frequency in Colombians (71.4%) is slightly smaller than that reported in Mexicans (79%) [10].

T334G and G699A were described as being in linkage disequilibrium in Japanese patients with renal failure [39]. Our results suggest that these SNPs are in linkage disequilibrium for the population of healthy individuals from Portugal but frequencies for these two SNPs are different in the case of Colombia and Mozambique, substantially so in the latter case.

Ser112Ala (T334G) is located in the putative transmembranar domain while Met233Ile (G699A) is located in the extracellular loop and their location can affect the substrate recognition characteristics of the SLCO1B3 protein. Therefore, these SNPs may be important for dosage adjustment in different populations.

The allelic frequency of SLCO1B3 G1564T (Gly522Cys) for the three populations analysed was around 5%, higher than the only report in a European population (1.9%) [6].

Taken together, our results suggest genetic similarities of all polymorphisms studied (except for SLCO1B1 C463A) between Portuguese and Colombian populations, but major differences in SNP frequencies between Portuguese/Colombian and Mozambican populations.

CONCLUSIONS

The liver is the main pharmacological target of many therapeutic compounds and it consists also in the major route of drug elimination. Genetic variants of influx transporters are of key relevance, as it has become evident that these proteins play a central role in drug disposition, contributing to variable efficacy and/or safety, as well as in the control of endogenous mediators such as cholesterol, bile acids or vasodilators.

For this reason it is important to characterize the genetic profile of populations from different ethnic backgrounds for adequate results in genotype/phenotype correlation studies and to aid in the prediction of differential susceptibility to adverse effects.

Populations from African and South American countries are substantially less characterized for pharmacogenetic variability, raising the issue that more information is needed before an adequate bridging of therapeutic dosages and regimens is possible for those populations.
The results obtained in this study contribute to that goal, given that the differential prevalence of variants for *SLC10A1*, *SLC22A1*, *SLCO1B1* and *SLCO1B3* determined in this study may contribute to variable disease risk and therapeutic outcome in individuals from Portugal, Mozambique and Colombia.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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ETHNIC DIFFERENCES IN THE PREVALENCE OF POLYMORPHISMS IN DRUG METABOLIZING ENZYMES INVOLVED IN CHOLESTEROL METABOLISM

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ABSTRACT

It is well known that drug disposition and response are greatly determined by the activities of drug metabolizing enzymes, which are polymorphic. Some of these polymorphisms are clinically relevant and presented an ethnic-dependent pattern of distribution. The characterization of the genetic distribution of different populations allows the selection of therapeutic options according with the genetic background, with the objective to avoid adverse reactions and inefficacy of the treatment. In this work we studied selected genetic polymorphisms in drug metabolizing enzymes in three different ethnic groups – Portugal, Mozambique and Colombia.

PCR-RFLP genotyping methods were developed for drug metabolizing enzymes, namely CYP7A1 (-203A>C, -346C>T, -496C>T, N233S, G347S), CYP27A1 (R164W, A169V, D273N, V400A) and CYP7B1 (-116C>G, R324H, 1774C>T) to characterize the allelic distribution of these polymorphisms among three different ethnic/geographic origins.

A total of twelve CYP7A1, CYP27A1 and CYP7B1 genetic variants were genotyped in a sample of 92 Portuguese, 151 Mozambican and 91 Colombian subjects.

The variants N233S in CYP7A1 and 1774C>T in CYP7B1 were not detected in any population studied. The promoter polymorphisms in CYP7A1 (-203A>C, -346C>T, -496C>T) had high frequency in the three ethnic groups. G347S (CYP7A1), R164W, A169V and V400A (CYP27A1) were present in a low frequency but with a similar distribution in the three ethnic groups. Significant differences were observed for D273N (CYP27A1),-346C>T (CYP7A1), -116C>G and R324H (CYP7B1).

Our results demonstrate a high variability of drug metabolizing enzymes between the different populations analyzed, indicating that, at least some of these polymorphisms are ethnic-specific.

Keywords: drug metabolizing enzymes, CYP7A1, CYP27A1, CYP7B1

Abbreviations: CYP: Cytochromes P450*; CYP7A1:* Cholesterol 7α-hydroxylase*; CYP27A1:* Sterol 27-hydroxylase*; CYP7B1:* Oxysterol 7α-hydroxylase*; FXR:* Farnesoid X Receptor*; HMG-CoA:* 3-hydroxy-3-methylglutaryl coenzyme A

INTRODUCTION

The main promise of Pharmacogenetics is the individualized therapy, giving the patient a dug selection and dosage according with his genetic profile. This kind of treatment should prevent adverse reactions or treatment failure and provide more effective and safe therapeutics for each person.

Although many polymorphic transporters and receptors have been associated with clinical consequences, drug metabolizing enzymes are the most likely reason for inter-individual variation in drug response (Roots *et al*, 2007).

Cytochromes P450 play a key role in drug response and also in the metabolism of endogenous compounds. Several polymorphisms have been described for these enzymes and their functional consequences may result in a moderate reduction, a lack or an increase of enzymatic activity.

Cardiovascular diseases are the most prevalent cause of death and morbidity in developed countries and the excess of cholesterol is a known factor for the progression of cardiovascular events. Cytochromes P450 are involved in cholesterol homeostasis, playing key roles in cholesterol metabolism, either in synthesis or elimination through biosynthesis of bile acids, the main products of cholesterol catabolism (Sarkis and Roman, 2004). Besides the role of CYPs in the homeostasis of endogenous substrates, these enzymes may be of crucial importance in the metabolism of drugs used to treat cardiovascular diseases (Siest *et al*, 2003). The cholesterol lowering drugs in use are mainly 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), fibrates, ezetimibe and bile-acid binding resins that act reducing the levels of cholesterol by different pathways (Schmitz and Lagmann, 2006).

Clinically relevant drugs are transported and metabolized by polymorphic proteins. Some of these SNPs are associated to altered function of the protein (Schmitz and Langman, 2005; Schmitz *et al*, 2001).

Cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme in the classical pathway for the synthesis of bile acids and its activity is highly variable among healthy individuals (Chiang, 2004). CYP7A1 is highly regulated by bile acids via Farnesoid X Receptor (FXR) (Lu, 2000). It has narrow substrate specificity for cholesterol, which makes this gene an important candidate in disorders of cholesterol and bile acids metabolism. In fact, some polymorphisms of CYP7A1 have been associated with gallstone disease and familiar hypertriglyceridemia (Cohen *et al*, 1992; Paumgartner and Sauerbruch, 1991; Angelin *et al*, 1987) and recently were associated with increased plasma LDL-cholesterol concentrations (Wang *et al*, 1998).

Sterol 27-hydroxylase (CYP27A1) and oxysterol 7α-hydroxylase (CYP7B1) are involved in bile acid synthesis in the alternative pathway (Tontonoz and Mangelsdorf, 2003).

CYP7B1 has high homology with CYP7A1 and it was hypothesized as the main function of this enzyme, the inactivation of oxysterols, protecting the liver from cholestasis (Chiang, 2004). Mutations in CYP7B1 are related with spastic paraplegia autosomal recessive type 5A (SPG5A), which is a neurodegenerative disorder characterized by a slow, gradual, progressive

weakness and spasticity of the lower limbs (Wilkinson *et al*, 2003; Tsaousidou *et al*, 2008). Absence of CYP7B1 is the cause of congenital bile acid synthesis defect type 3 (CBAS3), which is characterized by severe cholestasis, cirrhosis and liver synthetic failure (Setchell *et al*, 1998).

CYP27A1 is a ubiquitous enzyme involved in the 27-hydroxylation of cholesterol and in formation of potentially important regulatory oxysterols (Li *et al*, 2007), indicating a potential regulatory role for CYP27A1. Mutations in CYP27A1 were identified in patients with cerebrotendinous xanthomatosis, a rare sterol storage disorder characterized clinically by progressive neurologic dysfunction, premature atherosclerosis and cataracts (Guyant-Marechal *et al*, 2005).

As CYP7A1, CYP27A1 and CYP7B1 are also repressed by bile acids via nuclear receptors (Hylemon *et al*, 2009).

An evaluation of SNP frequencies among different populations with variable ethnic background will certainly be useful to characterize the changes in drug pharmacokinetics and predict differential susceptibility to possible adverse effects.

In the present study we examined the allelic frequencies of twelve CYP7A1, CYP27A1 and CYP7B1 SNPs, that may have an important role in drug metabolism, in populations from three different ethnic/geographic origins.

METHODS

Subjects. The populations under study consisted of 92 Portuguese, 151 Mozambican and 91 Colombian subjects. The populations from Mozambique and Portugal were described elsewhere (Cavaco *et al*, 2003; Cavaco *et al*, 2005). Colombian subjects were from the North-West region, mainly from Antioquia and Chocó Departments (62 men, 27 women). This study followed the recommendations of the Declaration of Helsinki, promulgated in 1964 (http://ohsr.od.nih.gov/helsinki.php3). Genomic DNA was extracted from whole peripheral blood, using the Qiamp DNA Extraction Kit (Qiagen, Hilden, Germany) or Chelex (BioRad) according to the manufacturer's protocols.

Pharmacogenetic analysis of polymorphic variants. Each of the twelve SNPs analysed in CYP7A1, CYP7B1 and CYP27A1 (NC_000008.10, NC_000008.10 and NC_000002.11, respectively) were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays.

Table 1 lists the primers employed in performing PCR for the analysed SNPs.

Table 1: Primers used in PCR

Gene	Polymorphism	Forward primer (5'→3')	Reverse Primer (5'→3')
CYP7A1	-203A>C -346C>T -496C>T	TGGCCTTGAACTAAGTCCACAGG	AGCACTGAAACATGAAGCAGC
	N233S	TCTTACAAGGCGGGACACACAGAA	AGTCGCTGGAATGGTGTTTGCTTG
	G347S	AGCAGAACTGAATGACCTGCCAGT	ATTCTACCCTGGCTGCAGCTTAAC
CYP27A1	R164W A169V	ATCTTTGTGCTGTTCCTCTGCGTC	AGTAGAAGAGTTGAGCCATGTCCG
	D273N	ACCTTCGTCAGATCCATCGGGTTA	ATGATCTCCAAGGACCAAGAGCCA
	V400A	TGAATCCAGAGCAGACTCCAGACA	AGTGGCAGAACACAAACTGGGTCT
CYP7B1	-116C>G	ACAAGCAAGCAGAGGGCCA	TTTGTCATTCAGCCTGGGAGACC
	R324H	AGGCTGTCCAATTGTTCTCTGGTG	GCACATCATTTAGGCTTTCTCTGG
	1774C>T	AGCCCATAGGACTAAACTACAGCC	GGGCTTTGTGACTAAGGACAAACTGG

Genomic DNA was added to PCR mixtures of 25µl consisting of 1× PCR buffer, 1-4mM $MgCl_2$ (Promega), 0.2mM dNTPs (Promega), and 1.5U of Go *Taq* Polymerase (Promega). PCR amplifications were conducted (Tpersonal Themocycler, Biometra) using one denaturation step at 94ºC for 2min, followed by 30-35 cycles of 94ºC for 30s, 40-55ºC for 30s and 72ºC for 1min and a final PCR extension of 3min at 72ºC.

After amplification by PCR, genotype assignment was performed by digestion of amplification products with specific restriction endonucleases (Table 2), separation by electrophoresis on 8% or 10% (W/V) poliacrylamide gels and visualized with ethidium bromide staining under UV light (AlphaImager, AlphaInnotech).

Table 2: Restriction Endonucleases used in PCR-RFLP

Gene	SNP	Fragment length of PCR product (bp)	Restriction Enzyme	Cleavage pattern (pb)
CYP7A1	-203A>C	370	MboI	A: 292+78 C:370
	-346C>T	370	DraI	C: 370 T: 213+157
	-496C>T	370	MaeII	C: 370 T: 342+28
	N233S	310	HpyCH4III	A: 310 G: 185+125
	G347S	256	BslI	G: 230+26 A: 256
CYP27A1	R164W	216	TspRI	C: 168+48 T: 90+78+48
	A169V	216	MspA1I	C: 126+90 T: 216
	D273N	261	BseGI	G: 153+108 A: 261
	V400A	226	HaeIII	T: 226 C: 173+53
CYP7B1	-116C>G	288	SacII	C: 200+88 G: 288
	R324H	197	DpnI	T: 197 A: 160+37
	1774C>T	253	Bpu10I	C: 114+139 T: 253

Statistical analysis. Fisher's exact test was used to assess Hardy-Weinberg equilibrium. Statistical analyses were performed using the Student's *t*-test and the χ^2 test.

All analyses were performed using the Primer software.

RESULTS

A total of twelve CYP7A1, CYP7B1 and CYP27A1 genetic variants were genotyped in a sample of 92 Portuguese, 151 Mozambican and 91 Colombian subjects. The allelic frequencies of CYP7A1, CYP7B1 and CYP27 in different ethnic groups are shown in table 3.

Table 3: CYP7A1, CYP27A1 and CYP7B1 allelic frequencies (%) in subjects of different ethnic groups

Gene	SNP	dbSNP ID	Portuguese (n=194)	95% CI	Mozambican (n=302)	95% CI	Colombian (n=182)	95% CI
CYP 7A1	-203A>C	rs3808607	41.8	35.85-55.80	52.6	27.74-53.74	40.7	34.83-54.74
	-346C>T	rs3808608	29.3	22.90-46.20	13.9*	6.46-15.97	24.2	18.15-35.94
	-496C>T	rs3824260	36.9	30.78-50.44	50	26.92-51.72	31.9	25.82-44.97
	N233S	rs8192874	0	0.00-4.81	0	0.00-2.98	0	0.00-4.81
	G347S	rs58192875	0.5	0.00-6.49	2.3	0.00-4.97	0.5	0.00-6
CYP 27A1	R164W	rs61733615	1.1	0.00-6.49	5.3	1.21-7.68	2.2	0.00-8
	A169V	rs59443548	2.7	0.72-9.55	4.6	1.21-7.68	1.1	0.00-6.49
	D273N	rs11559242	6.5	3.39-15.12	14.6*	5.97-15.74	17*	11.77-27.71
	V400A	rs1803361	1.6	0.13-8.08	4.3	1.00-6.80	2.2	0.13-8.06
CYP 7B1	-116C>G	rs6994347	4.3	1.36-11.00	14.9*	5.97-15.74	3.3	0.79-9.55
	R324H	rs59035258	3.8	1.36-11.00	0*	0.00-2.98	4.9	2.04-12.40
	1774C>T	rs8192907	0	0.00-4.81	0	0.00-2.98	0	0.00-4.81

* $p<0.001$

The variants N233S (CYP7A1) and 1774C>T (CYP7B1) were not detected between the individuals analysed from Portugal, Mozambique and Colombia. In contrast, the CYP7A1 promoter variants had a high frequency between the three populations studied. While, the frequencies for the -203C>T and -496C>T variants were similar between the three populations, the -346C>T variant was significantly lower for the population of Mozambique ($p<0.001$) compared with Portuguese and Colombian individuals. The frequency of G347S was similar between individuals from different ethnies, but with low allele frequency (0.5% for Portuguese and Colombian and 2.3% for Mozambican individuals).

For CYP27A1 the variants R164W, A169V and V400A had a low allelic frequency for the populations studied. The variant D273N was significantly more prevalent in Mozambican (14.6%, with $p<0.001$) and Colombian (17%, with $p<0.001$) individuals than in the Portuguese (1.6%) population.

For CYP7B1 the variant -116C>G had a low frequency in Portuguese (4.3%) and Colombian (3.3%) individuals, but a higher frequency among Mozambicans (14.9%, with $p<0.001$). The variant R324H had low frequency between Portuguese (3.8%) and Colombian (4.9%) populations, but wasn't found in Mozambican (0%, with $p<0.001$) individuals.

None of the genetic variants showed any significant deviation from Hardy-Weinberg equilibrium.

DISCUSSION

Drug metabolizing enzymes play a key role in cholesterol metabolism and may be important in the metabolism of some lipid-lowering drugs, such as some statins. Although, influx and efflux transporters are important for the disposition and efficacy of many drugs, CYP enzymes should have an important contribute for inter-individual variation in drug response.

As the response to a large range of drugs is highly variable, pharmacogenetic diversity may in part contribute to this variability, being important in the context of therapeutic efficacy and safety. An evaluation of SNP frequencies among different populations with variable ethnic background will certainly be useful to characterize the changes in drug pharmacokinetics and predict differential susceptibility to possible adverse effects.

Except for the polymorphism -203A>C (CYP7A1) and some variations of CYP27A1 found in some patients with cerebrotendinous xanthomatosis, there are no much information about CYP7A1, CYP27A1 and CYP7B1 polymorphisms. However these enzymes are extremely important in bile acid synthesis and its accumulation in the organism has harmful consequences for individual health, so it is important not only to know the genetic background of populations from different origins, but also to determine the functional consequences of these genetics variants for the enzyme activity.

We analyzed the frequencies of twelve SNPs in three CYP enzymes in individuals from Portugal, Mozambique and Colombia. From these SNPs, CYP7A1 N233S and CYP7B1 1774C>T were not detected in any of the three populations in study.

The variant N233S of the CYP7A1 is localized in exon 3 and CYP7B1 1774C>T is localized in exon 6, 3'-downstream region. The absence of these SNPs in the populations analysed may indicate that these mutations are localized in conservative regions of the gene and their substitution may lead to non-functional proteins.

The allelic frequency for CYP7A1 -203A>C among Caucasian range from 36.7-48% (Hofman *et al*, 2004; Hofman *et al*, 2005; Kajinami *et al*, 2004; Kajinami *et al*, 2005; Juzyszyn *et al*, 2008; Nakamoto *et al*, 2006). The estimate of 41.8% found in Portugal is in the range for a European/ Caucasian population. The allelic frequency obtained for Mozambicans (52.6%) is lower than reported by Nakamoto *et al* for an African (58.3%) or an Afro-American (58%) population, and for Colombians (40.7%) is significantly higher than for a Mexican-American (24%) population (Nakamoto *et al*, 2006). This discrepancy may be caused by differences in sample sizes between these two studies, but the most probable cause is the origin of the samples. Africa has different ethnic groups according with the geographic localization. The African subjects analysed by Nakamoto *et al* were from Yoruba of Ibadan, Nigeria, localized in Guinea Golf, West coast, while the individuals analysed in this study were from Mozambique, localized in East coast of Africa, which can explain the allelic frequencies observed. The high frequency determined in Colombians is consistent with the European (Spanish) ancestry of a wide percentage of the population.

CYP7A1 -203A>C was associated with LDL-cholesterol (Wang *et al*, 1998) and in patients with hypercholesterolemia -203A>C was associated with LDL-lowering response

(Kajinami et al, 2004), as well as, after treatment with statin therapy (Kajinami *et al*, 2004a). Therefore, this SNP contributes to the efficiency of statin treatment. Recently this SNP was also positively associated with subclinical atherosclerosis in healthy postmenopausal women (Lambrinoudaki *et al*, 2008) and it was considered a genetic risk factor for gallbladder carcinoma, but with a modest contribution in susceptibility to gallstone disease (Srivastava *et al*, 2008).

The allelic frequencies obtained to CYP7A1 -496C>T were high but similar between the three populations studied. The results obtained for Portugal (36.9%) and for Mozambique (50%) are in agreement with previous reports for a Caucasian (36.7%) and African (55%) population, respectively (Nakamoto *et al*, 2006).

The allelic frequency of CYP7A1 -346C>T was high for the three populations studied, but significantly lower for Mozambique (13.9%, with $p<0.001$).

The allelic frequency obtained for CYP7B1 -116C>G (4.3%) in Portuguese is in agreement with the frequencies obtained by Jakobsson *et al* for a Swedish population (4.04%) (Jakobsson et al, 2004). In the population of Mozambique the allelic frequency (14.9%) was significantly higher than the observed for Portugal and Colombia (3.3%). However, it wasn't found any reports for populations from these geographic points to compare with.

The genetic variants -203C>A, -346C>T and -496C>T are localized in the promoter region of the CYP7A1 gene and therefore it is much likely that these polymorphisms have functional significance. In fact, studies on the promoter region of CYP7A1 revealed several cell-specific enhancer elements (Molowa *et al*, 1992) and in another study the deletion of this region, in HepG2 cells, resulted in the 2.5 fold increase of basal promoter activity and this segment bound a negative regulatory protein of HepG2 cells (Cooper *et al*, 1997). These results suggested that polymorphisms in CYP7A1 promoter region may modulate transcriptional activity and consequently the rate of cholesterol catabolism and its flux through the body.

CYP7B1 -116C>G is localized in promoter region of the gene and it was associated with an altered promoter activity (Jakobsson *et al*, 2004). Besides, this polymorphism was considered ethnic-dependent, since significant differences in allele frequencies were observed for populations with distinct geographic localization (Jakobsson *et al*, 2004), which is in agreement with our observations.

To the best of our knowledge it is the first description of remain polymorphism analysed in this study, namely CYP7A1 (-346C>T, N233S, G347S), CYP27A1 (R164W, A169V, D273N, V400A), CYP7B1 (R324H, 1774C>T).

CYP7A1 G347S and CYP27A1 R164W, A169V and V400A were rare, with similar allelic distribution among populations.

The polymorphism G347S is localized in exon 4 of CYP7A1. Gly (G) is an important aminoacid for proper folding of the protein and it is known to be helix breaker and folding blocker of proteins. Hence the substitution of a Glycine in the protein can lead to conformational changes of CYP7A1.

The allelic frequency of CYP27 D273N for the Portuguese population (6.5%) was significantly slower than the observed for Mozambique (14.6%) and Colombia (17%) and it represents the only polymorphism in this study with a distribution significantly different from the Portuguese population.

The variants R164W and A169V are localized in exon 3, while the variants D273N and V400A are localized in exon 4 of CYP27A1. The variants R164W and A169V appear to be located in C-helix α, which is involved in conformational structure of the protein (Sawada *et al*, 2001). Arg residues (R) play important roles in proper folding of proteins as they can form salt bridges with Asp (D) and Glu (E) residues to stabilize the tertiary structure of proteins and, as these polymorphisms are near to conservative aminoacids, with important functions in folding and stabilization of the conformational structure of the protein, these genetic variations may be important to predict the correct folding of CYP27A1. In fact, more than 70% of the mutations reported in cerebrotendinous xanthomatosis are Arg, Gly or Pro polymorphisms, suggesting that these patients have conformational CYP27A1 changes.

D273N appears to be located on the third substrate recognition site and V400A in the fifth substrate recognition site sheet β1-4, which is involved in heme-binding (Sawada *et al*, 2001). These sites are very important for the functionality of CYP27A1, and although the polymorphisms do not lead to substitutions of very different aminoacids, the low frequency found for these SNPs suggest that they are localized in semi-conservative regions.

Alterations in protein aminoacids can lead to complete loss of its folding and consequently its structure, which can have drastic effects such as the elimination of enzyme active sites, creation of new binding sites or general disfunction

The allelic frequency for CYP7B1 R324H was similar between Portuguese (3.8%) and Colombian (3.3%), but significantly different from Mozambican ($p<0.001$), were the polymorphism wasn't found.

The genetic variant R324H is localized in exon 4 of CYP7B1. The low allelic frequency observed for this SNP may be explained by the fact that Arg (R) is involved in proper folding of proteins and can form salt bridges with Asp and Glu residues to stabilize the tertiary structure of the protein.

While polymorphisms in the promoter region can modify the degree of gene expression, polymorphisms in coding region may have a direct effect in enzymatic activity altering drug pharmacokinetics.

Our results suggest genetic similarities of all polymorphisms studied (except for CYP27A1 D273N) between Portuguese and Colombian populations, but major differences in SNP frequencies between Portuguese/Colombian and Mozambican populations. These results demonstrate that analyses of populations from different ethnic background are essential for adequate results in genotype/phenotype correlation studies and to predict differential susceptibility to possible adverse effects.

CONCLUSIONS

Pharmacogenetics constitutes the most important part of future molecular medicine. For that, molecular diagnostics along with molecular specified drug therapy will allow a safer and more efficient treatment.

We consider CYP7A1, CYP27A1 and CYP7B1 good candidates for pharmacognetic analysis of cholesterol-lowering therapy, since these CYP enzymes play key roles in cholesterol homeostasis and genetic variations in these genes most likely have a great impact on total body lipid metabolism. The characterization of variants in these genes among individuals from distinct geographic backgrounds will allow the development of new, more effective and safer drugs for each person. An evaluation of SNP frequencies among different populations with variable ethnic background will certainly be useful to characterize the changes in drug pharmacokinetics and predict differential susceptibility to possible adverse effects.

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INTER-ETHNIC VARIABILITY IN ABC TRANSPORTERS ABCB11, ABCG5 AND ABCG8

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ABSTRACT

Efflux transporters are known as key contributors to the process of drug metabolism. Polymorphisms of ABC transporters have been demonstrated to influence *in vitro* transport function and the pharmacokinetic profile of compounds and to confer susceptibility of several cholesterol associated diseases. Therefore, the pharmacogenetic analysis of ABC transporters might help to personalize and optimize drug therapy. Due to ethnic variability in polymorphism distribution, some ethnies may be at higher risk for adverse events, treatment failure or prevalence of pathologies.

The aim of this work was to study selected genetic polymorphisms in three efflux transporters, in individuals from distinct ethnic/geographic background, from Portugal, Mozambique and Colombia. PCR-RFLP genotyping methods were developed for a total of twelve SNPs in ABCB11 (BSEP), ABCG5 and ABCG8.

The variant G260D in ABCB11 was not detected in any population studied. High frequencies were determined for V444A (ABCB11), and T400K (ABCG8), with a similar distribution in individuals from the three populations. S194P (ABCB11) was rare in the ethnic groups of Mozambique and Colombia and absent in Portugal and R50C and T517S (ABCG5) were present in a low frequency in the three different ethnic groups. Significant differences were observed for M677V (ABCB11), G27A and Q604E (ABCG5) and D19H, C54Y and V632A (ABCG8). This is, to our knowledge, the first description of pharmacogenetic variability in influx transporters in Portuguese Caucasian, native Africans or Latin Americans.

Keywords: efflux transporters, SNP, ABCB11, ABCG5, ABCG8

Abbreviations: ABC: ATP-Binding Cassette; BSEP: Bile Salt export Pump; Receptor; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; PBC: primary biliary cirrhosis; PSC: primary sclerosing cholangitis; SNP: Single Nucleotide Polymorphism

INTRODUCTION

Pharmacogenetics and genomics have as main objective the individualized therapy, providing more effective and safe therapeutics as well prevent adverse reactions or treatment failure. This tailored therapy is based in the genetic profile of the patient, which allows the clinical to make a drug selection and dosage more efficiently.

In industrialized countries, cardiovascular diseases are the most prevalent cause of death and morbidity and the excess of cholesterol is in most causes the main factor for the progression of cardiovascular events. Therefore, it became important to study transporters involved in the excretion of bile acids and cholesterol, as well, drugs used in the treatment of cardiovascular diseases. The cholesterol lowering drugs in use are mainly 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), fibrates, ezetimibe and bile-acid binding resins that act reducing the levels of cholesterol by different pathways (Schmitz and Lagmann, 2006).

Some transporters and nuclear receptors were already associated with clinical consequences, and polymorphisms are indicated as the main reason for inter-individual variation in drug response (Roots *et al*, 2007). Clinically relevant drugs are transported and metabolized by polymorphic proteins. Some of these SNPs are associated to altered function of the protein (Schmitz and Langman, 2005; Schmitz *et al*, 2001).

To avoid intracellular accumulation of metabolites, an active transport is mediated by ABC (ATP-binding cassette) transporters, which control the absorption and elimination of endogenous and exogenous compounds. ABC transporters are a superfamily of transmembrane proteins which mediate transport of a wide variety of substrates across cellular membranes, process driven by the hydrolysis of ATP (Higgins, 1992). Several members of this superfamily have been associated to human diseases related with cholesterol alterations.

ABCB11 (BSEP) is involved in bile acids excretion (Lecureur e*t al*, 2000) while the efflux transporters ABCG5 and ABCG8 promote sterol and cholesterol export (Langheim *et al*, 2005). ABCG5 is a half transporter that forms a heterodimer with ABCG8 to become functional.

ABCB11 is highly regulated by bile acids via FXR/RXR (Wang *et al*, 1999) and PXR was also described as a potential regulator of ABCB11 (Koesters and Karpen, 2008). The regulation of ABCB11 gene expression by ligands and nuclear receptors is complex and points this gene as a critical component of the hepatocytes response to a variety of disease states and xenobiotics (Koesters and Karpen, 2008). In fact, several mutations in ABCB11 were associated with progressive familial intrahepatic cholestasis type 2 (PFIC 2) (Oude Elferink *et al*, 2006), which is characterized by severe jaundice, hepatomegaly, failure to thrive and pruritus, whereas liver histology shows portal inflammation and giant-cell hepatitis (Bezerra and Balistreri, 2000). This disease has a rapid progressive course and generally leads to cirrhosis and liver failure with a need for liver transplant within the first decade of life. Besides this phenotype, some mutations found in ABCB11 showed reduced transport activity when analysed in vitro, as result of decreased protein expression, altered membrane targeting and increased degradation by the proteossome (Wang *et al*, 2002a, b; Hayashi *et al*, 2005; Kagawa *et al*, 2008).

ABCG5 and ABCG8 are induced by LXR/RXR nuclear receptors. Increased levels of cholesterol increase ABCG5/8 levels through activation of LXR (Repa *et al*, 2002). Activation of LXR was associated with an increased biliary cholesterol secretion, decreased fractional cholesterol absorption and increased fecal neutral sterol excretion (Yu *et al*, 2003). Polymorhisms in both transporters, ABCG5 and ABCG8 are related with sitosterolemia, which is a rare autossomal recessive disorder, characterized by increased intestinal absorption of all sterols including cholesterol, plant and selfish sterols and decreased biliary excretion of dietary sterols into bile. Patients frequently develop tendom and tuberous xanthomas, accelerated atherosclerosis and premature coronary artery disease (Berge *et al*, 2000).

Considering that ABCB11, ABCG5 and ABCG8 respond to cholesterol (Dias and Ribeiro, in preparation), variability in drug response and in the transport of endogenous compounds, such as cholesterol and bile acids, may be explained by the interindividual and interethnic differences in these three ABC transporters. In the present study we examined the allelic frequencies of twelve ABCB11, ABCG5 and ABCG8 SNPs, that may have an important role in drug disposition, in populations from three different ethnic/geographic origins.

METHODS

Subjects. The populations under study consisted of 92 Portuguese, 151 Mozambican and 91 Colombian subjects. The populations from Mozambique and Portugal were described elsewhere (Cavaco *et al*, 2003; Cavaco *et al*, 2005). Colombian subjects were from the North-West region, mainly from Antioquia and Chocó Departments (62 men, 27 women). This study followed the recommendations of the Declaration of Helsinki, promulgated in 1964 (http://ohsr.od.nih.gov/helsinki.php3). Genomic DNA was extracted from whole peripheral blood, using the Qiamp DNA Extraction Kit (Qiagen, Hilden, Germany) or Chelex (BioRad) according to the manufacturer's protocols.

Pharmacogenetic analysis of polymorphic variants. Each of the twelve SNPs analysed in ABCB11 (BSEP), ABCG5 and ABCG8 were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays.

Table 1 lists the primers employed in performing PCR for the analysed SNPs.

Table 1: Primers used in PCR

Gene	Polymorphism	Forward primer (5'→3')	Reverse Primer (5'→3')
ABCB11 (BSEP)	S194P	AGAGCTCACATCTTAGTTCCCAAG	AGCCATGCCACATATGAAAGCCCA
	G260D	AGGCTTGATAACATCCCAAGGTGC	ACTCTGCCAGCTGTGCAGTCCCAG
	V444A	ACTTCTTGGTCATGGCTCTCAG	ACTTGATCTGCAATGCCAAC
	M677V	TTGACAAGACCTGGCAGTCTAC	TCTGCAGGACAAGTCCCGAG
ABCG5	G27A	TTACCTGACGCTGTAGGAGGCAT	AATTTGCCCAGCTTTGCTGCCTGT
	R50C	TTCCCAAGCTTACCTGAGCTTCCT	ACTGAAACACGTTAGGAGCCTGTC
	T517S	ACAGCAGAGCCACTACACTGTTGA	TTTCTAGGACGCTGGGCTTACATC
	Q604E	ACAGTCGGCAGCTTCACTTCCATT	CCTGAGATAAACCACACCTGACAC
ABCG8	D19H	AGGGCTGCCGAAAGGGGCCACTCCCAG	AGCCTGGGCAACAGAGCGAGACTCC
	C54Y	ACACCCTGGAGGTCAGAGACCTCGTC	TGACACCTATTGCACCTGACACG
	T400K	TGCAGTGGACCTGACCAGCATTGAC	ACGCCACCATAACTGGCTAC
	V632A	ACCATCGCGGTCTCAGGAGATAAA	ACGTAGTACAGGACCATGAAGCCA

Genomic DNA was added to PCR mixtures of 25µl consisting of 1× PCR buffer, 1-4mM $MgCl_2$ (Promega), 0.2mM dNTPs (Promega), and 1.5U of Go *Taq* Polymerase (Promega). PCR amplifications were conducted (Tpersonal Themocycler, Biometra) using one denaturation step at 94ºC for 2min, followed by 30-35 cycles of 94ºC for 30s, 40-55ºC for 30s and 72ºC for 1min and a final PCR extension of 3min at 72ºC.

After amplification by PCR, genotype assignment was performed by digestion of amplification products with specific restriction endonucleases (Table 2), separation by electrophoresis on 8% or 10% (W/V) poliacrylamide gels and visualized with ethidium bromide staining under UV light (AlphaImager, AlphaInnotech).

Table 2: Restriction Endonucleases used in PCR-RFLP

Gene	SNP	Fragment length of PCR product (bp)	Restriction Enzyme	Cleavage pattern (pb)
ABCB11 (BSEP)	S194P	428	BseNI	T: 255+173 C: 428
	G260D	736	DpnI	G: 736 A: 392+344
	V444A	724	HaeIII	C: 463+261 T: 724
	M677V	356	BspMI	A: 356 G: 189+167
ABCG5	G27A	176	MwoI	G: 108+68 C:176
	R50C	269	BtgI	C: 135+134 T: 269
	T517S	156	DdeI	C: 93+63 G: 156
	Q604E	282	TspRI	C: 196+86 G: 282
ABCG8	D19H	394	BseLI	G: 368+26 C: 394
	C54Y	195	XmiI	A: 167+28 G: 195
	T400K	623	MseI	C: 328+295 A: 623
	V632A	206	NcoI	T: 206 C: 122+84

Statistical analysis. Fisher's exact test was used to assess Hardy-Weinberg equilibrium. Statistical analyses were performed using the Student's *t*-test and the χ^2 test.

All analyses were performed using the Primer software.

RESULTS

A total of twelve ABCB11, ABCG5 and ABCG8 genetic variants were genotyped in a sample of 92 Portuguese, 151 Mozambican and 91 Colombian subjects. The allelic frequencies of ABCB11, ABCG5 and ABCG8 in different ethnic groups are shown in table 3.

Table 3: ABCB11, ABCG5 and ABCG8 allelic frequencies (%) in subjects of different ethnic groups

Gene	SNP	dbSNP ID	Portuguese (n=184)	95% CI	Mozambican (n=302)	95% CI	Colombian (n=182)	95% CI
ABCB11 (BSEP)	S194P		0	0.00-4.81	2	0.00-4.97	1.1	0.00-6.49
	G260D		0	0.00-4.81	0	0.00-2.98	0	0.00-4.81
	V444A	rs2287622	58.2	52.83-72.21	61.6	32.29-68.74	52.7	47.40-67.21
	M677V	rs11568364	4.3	1.36-11.00	13.2**	4.95-14.19	6.6	3.39-15.12
ABCG5	G27A	rs56204478	18	12.66-28.91	9.3**	2.00-11.02	8.3*	4.25-16.45
	R50C	rs6756629	9.8	5.83-19.04	8.3	2.53-10.20	6	2.75-13.78
	T517S	rs17031672	9.8	5.83-19.04	8	2.50-10.18	12.7	8.31-22.83
	Q604E	rs6720173	16.8	11.77-27.71	28.5*	12.58-35.10	31.3*	24.84-43.86
ABCG8	D19H	rs11887534	7.6	4.25-16.45	1.9*	0.00-4.97	5.5	2.75-13.78
	C54Y	rs4148211	38.5	32.79-52.60	18.9*	8.07-27.78	31.9	25.82-44.97
	T400K	rs4148217	18.5	13.56-30.10	21.9	9.69-22.01	28.6	22.90-41.62
	V632A	rs6544718	23.9	18.15-35.94	8.2*	2.53-10.19	20.9	15.38-32.46

** p<0.001
* p<0.05

The variant G260D (ABCB11) was not detected between the individuals analysed from Portugal, Mozambique and Colombia, while the variant S194P (ABCB11) was not detected in the Portuguese population and were rare in Mozambicans and Colombians (2 and 1.1%, respectively). In contrast, the variant V444A had a high frequency between the three populations studied and the M677V variant was significantly higher for the population of Mozambique (p<0.001) compared with Portuguese and Colombian individuals.

For ABCG5 the variants G27A and Q604E were significantly different (p<0.001) between the Portuguese population and the other two ethnies analysed. In the case of G27A, this variant was more prevalent in the Portuguese population (18%) while the variant Q604E had higher frequency between Mozambicans (28.5%) and Colombians (31.3%). The variants R50C and T517S had similar allelic frequency between Portuguese, Mozambican and Colombian individuals.

For ABCG8 the variants D19H, C54Y and V632A were significantly less prevalent (1.9%, 18.9% and 8.2%, respectively) in the population of Mozambique (p<0.001) compared with the Portuguese population, while the variant T400K had similar distribution between the three populations.

None of the genetic variants showed any significant deviation from Hardy-Weinberg equilibrium.

DISCUSSION

ABC transporters are involved in the disposition and efficacy of many drugs such as statins (Rodrigues *et al*, 2009a). Therefore, efflux transporters affect the extent of oral bioavailability and the rate of absorption of these compounds (Rodrigues *et al*, 2009). Polymorphisms of ABCB11, ABCG5 and ABCG8 have been associated with cholesterol related diseases (Oude Elferink *et al*, 2006; Berge *et al*, 2000). As the response to a large range of drugs is highly variable, pharmacogenetic diversity may in part contribute to this variability, being important in the context of therapeutic efficacy and safety. An evaluation of SNP frequencies among different populations with variable ethnic background will certainly be useful to characterize the changes in drug pharmacokinetics and predict differential susceptibility to cholesterol related diseases.

We analyzed the frequencies of twelve SNPs in three ABC transporters in individuals from Portugal, Mozambique and Colombia.

Mutations in BSEP (ABCB11) are associated to hereditary forms of liver disease, as they lead to functional defects of BSEP-mediated canalicular bile salt secretion (Pauli-Magnus *et al*, 2005). So far, more than 30 mutations in ABCB11 have been identified in patients with BSEP deficiency syndrome (Pauli-Magnus *et al*, 2005). Although inherited mutations in ABCB11 are very rare, acquired forms of cholestasis, such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), cholestasis associated with pregnancy or drug-induced cholestasis are more frequent (Pauli-Magnus and Meier, 2005). The susceptibility of some patients to BSEP inhibition can be explained by genetic background, enhancing the importance of genetic-ethnic studies. From the analysed SNPs, S194P (ABCB11) was not detected in any of the three populations in study and G260D (ABCB11) was not detected in the Portuguese population, which in both cases, is in agreement with Pauli-Magnus *et al* (Pauli-Magnus *et al*, 2004a). S194P, G260D, V444A and M677V are located in the exons, 7, 8, 13 and 17, respectively of ABCB11. S194P is located in intracellular loop and it seems to be PSC-specific, while G260 is located in the transmembrane region and it is PBC-specific (Pauli-Magnus *et al*, 2004a). To the best of our knowledge, this is the first description of S194P and G260D for Africans and Hispanics.

The variants A444V and M677V are also located in intracellular loop of BSEP. Alignment of mammalian ABCB11 shows that the S194P, G260D and V444A variants are in codons for an evolutionary conserved amino acid (Pauli-Magnus *et al*, 2004a). V444A was considered a usual polymorphism in the western population, which is in agreement with the high allelic frequencies obtained in this study and when compared with another descriptions (Meier *et al*, 2006; Lang *et al*, 2006). However, some studies demonstrated that this variation may be associated with intrahepatic cholestasis of pregnancy (Pauli-Magnus *et al*, 2004b; Keitel *et al*, 2006) and with drug-related cholestasis (Lang *et al*, 2007). This is a consequence of the low BSEP expression level found in individuals with the 444A allele (Stieger *et al*, 2007). Individuals with low or very low expression of ABCB11 could be at risk to develop acquired forms of cholestasis, such as by drugs or pregnancy. The variant M677V was less frequent than V444A but its distribution was

ethnic specific ($p<0.001$ to Mozambique), which is in agreement with others authors (Pauli-Magnus *et al*, 2004a; Meier *et al*, 2006; Lang *et al*, 2006).

Mutations in ABCG5/G8 transporters are associated to sisterolemia and polymorphisms in these genes can be predicted to influence individual response to diet or drugs that could lead to increased disease risk (Rudkowska and Jones, 2008). Polymorphisms in these two transporters might contribute to the genetic variation in plasma lipid levels and in cholesterol saturation of bile and as they are also involved in the biliary secretion of cholesterol, they are the most susceptible for gallstone formation (Wittenburg *et al*, 2005).

For ABCG5 transporter, the polymorphisms studied in this work were G27A, R50C, T517S and Q604E. The variants R50C and T517S had a similar distribution among the three populations analysed, while G27A had significantly lower allelic frequency between Mozambicans and Colombians when compared with the Portuguese population. The allelic distribution of the variant Q604E was lower for the Portuguese population and significantly higher for Mozambique and Colombia. However, these distributions among different ethnies are in agreement with another reports to Caucasians (Iwona and Jones, 2008; Hubacek *et al*, 2004; Thompson *et al*, 2005; Plat *et al*, 2005), Afro-Americans (Thompson *et al*, 2005) and Hispanic (Thompson *et al*, 2005), respectively. Several studies have tried to demonstrate the effects of the mutation Q604E in cholesterol metabolism. According with Weggemans *et al* carriers of the wild-type allele had lower baseline plasma total cholesterol concentrations (Weggemans *et al*, 2002). However, other authors found contrary results (Herron *et al*, 2006) or were unable to observe associations between Q604E polymorphism and plasma lipid concentration following dietary or drug intervention (Berge *et al*, 2002; Gylling *et al*, 2004; Kajinami *et al*, 2004).

Among the SNPs analysed for ABCG8, only T400K has a similar distribution between the three populations. D19H, C54Y and V632A had a significantly lower expression in Mozambicans than in Portuguese and Colombians, whose allelic frequency was close. The distributions obtained for these polymorphisms are in agreement with other authors for Caucasians (Iwona *et al*, 2008; Hubacek *et al*, 2004; Thompson *et al*, 2005; Plat *et al*, 2005; Koeijvoets *et al*, 2008), Afro-Americans (Thompson *et al*, 2005) and Hispanic (Thompson *et al*, 2005), respectively. According with Hubacek *et al*, ABCG8 T400K and C54Y genotypes influence plasma total cholesterol in a gender-specific manner following dietary intervention (Hubacek *et al*, 2004). In addition, these polymorphisms may increase the risk of gallstone disease (Gruhage *et al*, 2007).

The polymorphisms Q604E (ABCG5) and D19H, C54Y, T400K and V632A (ABCG8) had been associated with several factors affecting cholesterol metabolism, such as baseline cholesterol levels, cholesterol absorption or responsiveness to dietary intervention, indicating a crucial role for these two transporters in cholesterol homeostasis (Iwona *et al*, 2008). Carriers of D19H mutant allele present an up-regulated synthesis of cholesterol (Berge *et al*, 2002; Gylling *et al*, 2004). This can result in a higher efficiency of statin therapy, which is in agreement with the results obtained by Kajinami *et al*, which associate the mutated allele of D19H to a greater plasma LDL-cholesterol reduction after atorvastatin treatment, in comparison with the wild-type allele (Kajinami *et al*, 2004). Besides, Kajinami *et al*, also demonstrated that these reductions in

LDL-cholesterol were higher in individuals with the wild-type allele of CYP7A1 (Kajinami *et al*, 2004). Also, the mutant allele of D19H and T400K might help to predict gallstone disease risk, as the mutant allele of D19H was associated with gallstone disease, suggesting that this variant may confer a more efficient transport of cholesterol into bile (Buch *et al*, 2007) and male carriers of the mutant allele of T400K were more susceptible for gallstone disease, compared with wild-type carriers, with no association was found in female carriers (Wang *et al*, 2007).

Our results suggest genetic similarities of almost polymorphisms studied (except for ABCG5 G27A and Q604E) between Portuguese and Colombian populations, but major differences in SNP frequencies between Portuguese/Colombian and Mozambican populations, in particular for ABCG8 variants analysed here. These results enhance the importance of genetic analysis of populations from different ethnic backgrounds to characterize adequately genotype/phenotype relations, to avoid possible adverse events to therapeutics and to develop optimal and individualized diets to decrease blood lipids and decrease the risk of cholesterol associated diseases.

CONCLUSIONS

Genetic variants of ABC-transporters are important as it has become evident that these proteins play a key role in the disposition and efficacy of many drugs, as well as in the control of endogenous mediators such as cholesterol, bile acids o vasodilators. They also are important in the prediction of the susceptibility of several cholesterol related diseases. Therefore, these polymorphic efflux transporters lead to inter-individual variability in disease risk, as well as in the risk of adverse events or treatment failure being of extreme importance the characterization of these variants among individuals from distinct/geographic backgrounds. Knowing the genetic background of a population allows developing new, more effective and safe therapeutics for each person and to predict the evolution of several disease conditions allowing its preventive treatment and diminishing the severity of the disease. An evaluation of SNP frequencies among different populations with variable ethnic background will certainly be useful to characterize the changes in drug pharmacokinetics and predict differential susceptibility to possible adverse effects and the susceptibility to several cholesterol related pathologies.

Our results demonstrate variability of ABC transporters between the different populations analyzed, indicating that, at least, some SNPs are ethnic-specific.

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2. Expression profile of genes involved in drug metabolism and transport in response to cholesterol

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Paper IV – The expression of the solute carriers NTCP and OCT-1 is regulated by cholesterol in HepG2 cells. Fundamental & Clinical Pharmacology (2007) 21: 445-450

Paper V – Effect of cholesterol on the expression of Solute carriers, Cytochrome P450 enzymes and ABC transporters in HepG2 and Caco-2 cells – (Manuscript)

ORIGINAL ARTICLE

The expression of the solute carriers NTCP and OCT-1 is regulated by cholesterol in HepG2 cells

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ABSTRACT

Drug disposition and response are greatly determined by the activities of drug-metabolizing enzymes and transporters. While the knowledge in terms of CYP enzymes and efflux ABC transporters (such as MDR1, P-glycoprotein) is quite extensive, influx transporters are increasingly being unveiled as key contributors to the process of drug disposition. There is little information on the regulation of these proteins in human cells, especially as regards the effect of endogenous compounds. In this study, we analysed the expression of CYP3A4 and three uptake transporters NTCP (SLC10A1), OATP-A/OATP1A2 (SLCO1A2) and OCT-1 (SLC22A1) in HepG2 cells following treatment with cholesterol. While CYP3A4 and OATP1A2 expression was unaffected, cholesterol treatment led to increased levels of NTCP and OCT-1 mRNAs. Alterations in the functional characteristics and/or expression levels of drug transporters in the liver may conceivably contribute to the variability in drug oral bioavailability often observed in the clinical settings.

INTRODUCTION

Cardiovascular diseases are the major causes of death and morbidity in developed countries. Several risk factors, such as high cholesterol levels, have been associated with cardiovascular diseases but the molecular mechanisms underlying the alteration of homeostasis are still far from being completely characterized.

The management of cardiovascular diseases is critically dependent on lipid-lowering therapy. Different classes of drugs are available for this purpose, such as statins, cholesterol absorption inhibitors, bile acid resins, fibrates and nicotinic acid that act by reducing the levels of cholesterol by distinct pathways [1]. The response to these drugs is highly variable and pharma cogenetic diversity may in part contribute to this phenomenon. However, therapeutic efficacy may also depend on the relative levels of the metabolizing enzymes and transporters that act on cardiovascular drugs.

The liver, the main detoxification organ in the body, extracts drugs from blood via specialized membrane transporters, making them available for biotransforma-tion by metabolizing enzymes. Phase I and II enzymes modify the substrate, generally increasing its solubility and facilitating its elimination. Metabolic products are then excreted from the cell through the action of a number of ATP-dependent membrane transport proteins.

The study of the changes that occur in this metabolic system in response to alterations of the steady-state concentrations of endogenous compounds involved in the development of disease can be extremely important, not only to obtain adequate knowledge on drug phar-macokinetics but also to predict occurrence of potential adverse drug interactions.

Cholesterol plays an essential role in mammalian cells and its homeostasis is maintained by a delicate balance between cholesterol input and output pathways. Bile acids are important regulators of cholesterol homeostasis by inhibiting hepatic cholesterol metabolism into bile acids or

by enhancing uptake of dietary cholesterol. Thus, the levels of bile acids and cholesterol are linked and tightly controlled by nuclear receptor regulation of both choles terol catabolism and absorption [2].

The effects of cholesterol itself on the expression of genes involved in the metabolism and transport of endogenous and exogenous compounds are however poorly understood. Cytochrome P450 3A4 (CYP3A4) mediates the conversion of cholesterol to 4b-hydroxy-cholesterol [3], being also the key enzyme in the biotransformation of a wide variety of drugs to more polar derivatives, affecting their pharmacokinetic and pharmacological activities [4].

NTCP (also known as SLC10A1) is the basolateral (sinusoidal) Na_+-bile acid co-transporter that mediates the hepatic uptake of bile acids, being also responsible for the transport of drugs, including statins [5–7]. Organic anion-transporting polypeptides (OATPs) are basolateralplasma membrane transport proteins that mediate the sodium-independent transport of a wide range of amphipathic organic compounds including bile salts, organic dyes, steroid conjugates, thyroid hormones, anionic oligopeptides and numerous other drugs [8].

Within this family of transporters, OATP1A2 (previously called OATP-A, OATP, OATP1 and encoded by the SLCO1A2 gene, previously called SLC21A3) was shown to be expressed in several tissues, including the brain, liver, kidney, testis, prostate and fetal lung [9,10] and to mediate transport of a broad range of substrates from endogenous (e.g. bile acids, steroid hormones) to drugs (eg fexofenadine, ouabain and other anionic drugs, bulky organic cations, opioid peptides, lipid-lowering drugs) [11–14].

The organic cation transporter 1 (OCT1, SLC22A1) is another transporter protein, located in the basolateral membrane of several tissues, primarily in the liver, and to a lesser extent in the heart, skeletal muscle, intestinal and kidney [15]. This transporter mediates the uptake of a variety of endogenous compounds (e.g. noradrenaline, acetylcholine) and cationic drugs, such as cimetidine, clonidine, procainamide, desipramine, protease inhibitors, phenformin, midazolam and acyclovir [16–18].

Not much is known about the regulation of individual uptake transporters. In the present study we examined, in human hepatocarcinoma HepG2 cells, the effect of cholesterol on the expression of selected human genes involved in the metabolism and transport of endogenous and exogenous compounds: CYP3A4, SLC10A1, SLC22A1 and SLC21A3.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line HepG2 was maintained in Dulbecco's modified medium (DMEM) supplemented with 10% fetal bovine serum, 1% glutamine penicillin strep-tomycin, and 1% non-essential amino acids at 37 °C in a humidified 5% CO_2, 95% air atmosphere.

Cell treatments

Before treatment, cells were sequentially adapted to a serum-free environment. HepG2 cells were treated with 1–20 ug/ml cholesterol (Sigma-Aldrich Quımica S.A. Madrid, Spain). Ethanol was used as control vehicle.
Treatment durations were 0, 2, 4, 8, 12 and 24 h.

RNA isolation

Total RNA was isolated from HepG2 cells using a modification of the guanidine tiocyanate method [19].
The RNA was then purified from contaminating genomic DNA by incubation with DNase I (3 U/100IL RNA; Promega Biotech Iberica, SL, Madrid, Spain) at 37 °C for 30 min, followed by heat inactivation of the enzyme at 55 °C for 10 min.

Primers

Gene-specific primers were used for RT-PCR amplifica-tion as indicated in Table I. Primers for human ribosomal RNA 18S were used as a positive control for efficiency of RNA isolation and cDNA synthesis.

Table I Primers used in the RT-PCR assays.

Gene	Accession number	Forward primer (5' →3')	Reverse primer (5' →3')
18S	NC_000024.8	TGAAACTGCGAATGGCTCAT	CGACTACCATCGAAAGTTGA
CYP3A4	NC_000007.12	GGATCCATTCTTTCTCTCAAT	GTATCTTCGAGGCGACTTTC
OCT1/SLC22A1	NC_000006.10	TAATGGACCACATCGCTCAA	AGCCCCTGATAGAGCACAGA
OATP1A2/SLCO1A2	NC_000012.10	TGGGGAACTTTGAAATGTGG	AAGGCTGGAACAAAGCTTGA
NTCP/SLC10A1	NC_000014.7	GGGACATGAACCTCAGCATT	CGTTTGGATTTGAGGACGAT

Reverse transcriptase-polymerase chain reaction
RNA (400 ng) was transcribed by reverse transcriptase (50U, Invitrogen, Barcelona, Spain) for 50 min at 37°C. The reverse transcriptase was inactivated by heating to 70°C for 15 min. PCR was performed in the presence of 1–4 mM $MgCl_2$, 1x PCR buffer (Fermentas, Vilnius, Lithuania), 0.2 mM dNTPs, 2–4 pmol/lL of forward and reverse primers and 1.5U Taq polymerase (Fermentas). PCR amplifications were conducted using one denatura- tion step at 94°C for 2 min, followed by 40–45 cycles of 92.5°C for 30 s, 40–55°C for 45 s and 72°C for 1 min and final PCR extension of 3 min at 72°C (Eppendorf Mastercycler; Eppendorf Iberica, Madrid, Spain). Relative quantification of amplification products was performed by densitometric analysis (Alpha Imager; Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical analysis
Results are expressed as mean ± SD of three assays per condition, after normalization with the levels determined for the 18S rRNA.

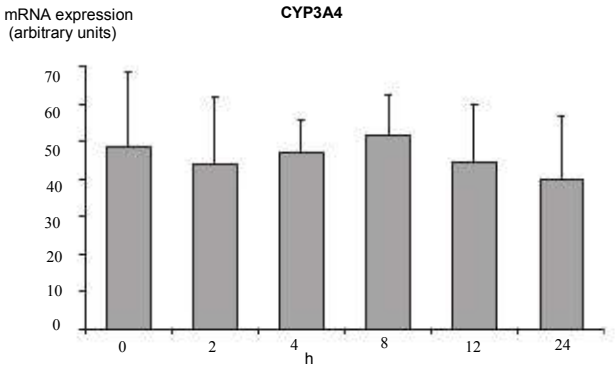

Figure 1 Effect of cholesterol on CYP3A4 mRNA expression in HepG2 cells. Cell culture media was supplemented with 5 ug/mL cholesterol for different periods as indicated. CYP3A4 mRNA relative levels were assessed by RT-PCR analysis using specific primers as described in Table I. Results are expressed as relative mRNA expression normalized to internal control 18S mRNA. Error bars represent the standard deviation from the mean of triplicate assays of an individual experiment.

RESULTS

We aimed to evaluate the effect of cholesterol on the expression of CYP3A4 and the influx transporters SLC10A1/NTCP, SLC21A3/OATP1A2 and SLC22A1/ OCT-1 in human HepG2 cells. Different cholesterol concentrations were tested in this study, ranging from 1 to 20 ug/mL, for different treatment periods from 2 to 24 h. No significant change was observed in the relative levels of CYP3A4 mRNA in HepG2 cells after cholesterol treatment (Figure 1).

OATP1A2 mRNA expression was slightly increased after 4 and 8 h of treatment, but this change did not reach statistical significance (Figure 2). The expression of both CYP3A4 and OATP1A2 mRNAs was not significantly altered regardless of the cholesterol concentration used (data not shown).

NTCP and OCT-1 mRNA levels were significantly induced ($P < 0.05$) in response to cholesterol treatment (5 lg/mL) during 8 h (Figure 2). The induction effect was observed at the same time point for all concentrations tested, with the maximal induction being observed for the concentration 5 ug/mL.

DISCUSSION

During the past decade there was an increased awareness of the critical interplay between drug-metabolizing enzymes and transporters as determinants of drug disposition and response. Several drug-metabolizing enzymes and transporters are able to act on both endogenous and exogenous substrates, constituting a potential focus of interference between specific physiological and pathological states and the clearance of xenobiotic substrates such as therapeutic drugs.

The effects of cholesterol on gene expression are far from being completely understood, namely its effect on drug metabolism and transport. We evaluated the pattern of expression in HepG2 of genes involved in the biotransformation of both cholesterol and therapeutic drugs, as well as in the transport of endogenous and exogenous substances. While CYP3A4 and OATP1A2 levels were not affected by cholesterol treatment, a significant increase in the expression of NTCP and OCT-1 was observed.

CYP3A4 is one of the major drug-metabolizing enzymes in human liver, mediating the conversion of several drugs used in the management of cardiovascular diseases, such as antidyslipidaemic drugs (e.g. simvastatin [20]) and antiarrythmic drugs such as diltiazem [21]. CYP3A4 expression is known to be regulated by a wide variety of exogenous stimuli, that act through key regulators such as the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) [22]. The effect of endogenous compounds on CYP3A4 gene expression has not been studied much.

In humans, there is indirect evidence that CYP3A induction occurs in response to cholestasis. Increased excretion of 6b-hydroxylated bile acids – a marker of CYP3A activity – occurs in cholestatic patients [23].

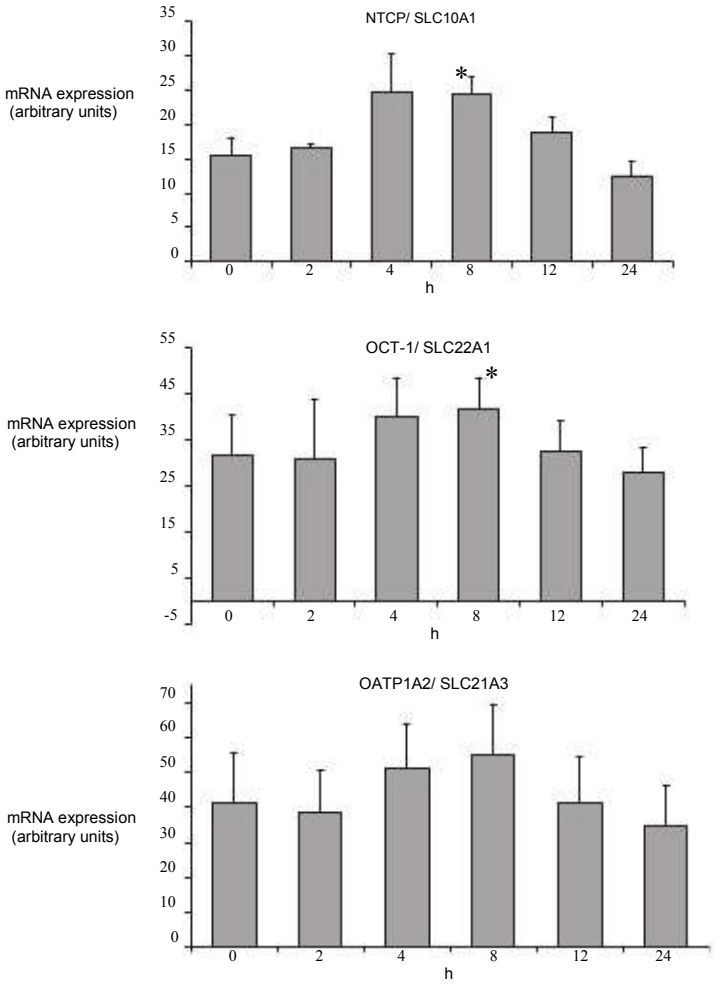

Figure 2 Effect of cholesterol on the expression of sinusoidal influx drug transporters in HepG2 cells. Cells were treated with 5 ug/mL cholesterol for different periods as indicated. mRNA relative levels for NTCP/SLC10A1, OCT-1/SLC22A1 and OATP-A/OATP1A2/SLC21A3 were evaluated by RT-PCR analysis using specific primers as described in Table I. Results are expressed as relative mRNA levels normalized to the internal control 18S mRNA. Error bars represent the standard deviation from the mean of triplicate assays of an individual experiment. *P < 0.05.

Moreover, while hepatic CYP concentrations are in general decreased in human livers with biliary cirrhosis, CYP3A4 expression is relatively preserved [24], suggesting that CYP3A induction occurs as a defence against bile acid accumulation [25]. However, induction seems to be independent of increased concentrations of lithocholic acid, a putative PXR ligand, as this hydrophobic secondary bile acid does not accumulate in a rodent model of cholestasis, suggesting that another agent is involved [25].

In the present study, CYP3A4 expression in HepG2 cells was shown to be unaffected by cholesterol. The reasons for this discrepancy are unclear, but may be related to differential cholesterol metabolism in HepG2 when compared to liver [26]. The expression levels and function of drug transporters are under the control of various physiological and exogenous stimuli. However, most of the information available concerning regulation of influx transporters is restricted to rodent proteins.

All the transporters analysed were detected in untreated HepG2 cells, in accordance with previous reports [27,28]. Cholesterol treatment led to an increase in expression, more evident in the case of NTCP and OCT-1 mRNAs. In rat liver, uptake of bile salts and other organic anions at the basolateral membrane is markedly impaired in cholestasis, in part because of the downregulation of Ntcp expression [29]. Accordingly, a similar decrease in NTCP expression was reported in percutaneous liver biopsy samples of patients with cholestatic liver disease [5].

NTCP has been shown to be downregulated by bile acids, through the action of farnesoid X receptor (FXR). The underlying mechanism involves the induction of the repressor short heterodimer partner (SHP), which interferes with the activity of the RAR/RXR heterodimer that controls the expression of the rat Ntcp gene [30].

Binding of the RAR:RXR complex is also downregulated by the inflammatory cytokine interleukin (IL)-1b, explaining downregulation of the rat Ntcp promoter secondary to IL-1 b exposure in HepG2 cells [31]. The reduction in expression of Ntcp in rat liver in ethinylestradiol-induced cholestasis has been associated with decreased binding of hepatocyte nuclear factor 1 (HNF1) and CCAAT/enhancer binding protein (C/EBP) [29]. In much the same way, endotoxin-induced cholestasis and toxic liver injury lead to reduced Ntcp transcription because of reduced binding of transactivators such as HNF-1, C/EBP and RAR/RXR heterodimer [32].

We observed an increase in NTCP levels brought upon by cholesterol, which seems to be one of the few known instances of positive regulation of this gene. Another example of positive regulation of NTCP expression was previously reported in response to prolactin via activation of the transcription factor STAT5 [33].

Cholestasis has also been shown to affect expression of OATPs. The reduction in expression of Oatps in rat liver in ethinylestradiol-induced cholestasis has been shown to be associated with decreased binding of PXR [29]. This is is agreement with data from PXR null mice, suggesting that PXR acts in the coordinated

control of both oatp2 and cyp7a1, the key enzyme in bile acid synthesis [34].

Transcription of liver OATPs has also been shown to be dependent on HNF1alpha, in accordance with the presence of a HNF-1 binding site in the regulatory region of SLC21A3 [35]. Bile duct ligation in the rat, an established model of obstructive cholestasis, resulted in profound downregulation of the organic cation transporter Oct1 mRNA and protein hepatic levels and in the impairment of the uptake of prototype Oct1 substrates.

In contrast, Oct1 gene expression in the kidneys was not impaired and even appeared to be upregulated transiently [36]. This suggests an involvement of tissue-specific transcription factors as part of a very complex regulatory mechanism. Transport mediated by human OCT1 has also recently been demonstrated to decrease upon activation of protein kinase A [37].

Taken together, our results point to an increased expression of uptake transporters triggered by cholesterol. To our knowledge this is the first description of such a study concerning membrane uptake transporters. These transporters are known to be repressed by bile acids. In rat liver, the majority of cholesterol is metabolized to bile acids that can repress some target genes when they are at high levels in the cell. In HepG2 cells low levels of bile acids should be present because of reduced metabolism. Although we cannot exclude an indirect action via oxysterol binding and activating of the liver X receptor (LXR), our observations could be the result of a direct action of cholesterol. As cholesterol has been suggested as the natural ligand of the retinoic acid receptor-related orphan receptor alpha (RORa) [38], it is conceivable that this transcription factor may contribute to the mechanism underlying induction by cholesterol in HepG2 cells. The existence of putative ROR-responsive elements in the 5'-flanking regions of SLC10A1, SLC21A3 and SLC22A1 lends support to this hypothesis. Studies are underway, aiming at the identification of the molecular mechanisms underlying the observed NTCP and OCT1 induction by cholesterol.

CONCLUSIONS

Carrier-mediated hepatic uptake plays a key role in the disposition and efficacy of many drugs. Some transporters are highly expressed in the liver in which they may be involved in hepatic clearance of substrates from the portal circulation. Additional functional studies are necessary to establish if changes in the expression of drug transporters in the liver and/or intestine in a pathological situation – such as hypercholesterolaemia – may contribute to the variability in drug oral bioavailability often observed in clinical settings.

ACKNOWLEDGEMENTS

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EFFECT OF CHOLESTEROL ON THE EXPRESSION OF SOLUTE CARRIERS, CYTOCHROME P450 ENZYMES AND ABC TRANSPORTERS IN HEPG2 AND CACO-2 CELLS

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ABSTRACT

Objectives: Drug disposition and response is greatly determined by the activities of drug metabolizing enzymes and transporters. There is increasing evidence that drug metabolizing enzymes, influx and efflux transporters work in a coordinated fashion on the process of drug disposition. So it became important to study the regulation of these proteins in human cells, in which concerns the effect of endogenous compounds, such as cholesterol.

Methods: In this study, we analysed the expression of a wide range of membrane influx (SLC10A1, SLC22A1, SLC22A7, SLC01A2, SLCO1B1, SLCO1B3, SLCO2B1) and efflux transporters (ABCA1, ABCB11, ABCC2, ABCC3, ABCC6, ABCG2, ABCG5, ABCG8) and drug metabolizing enzymes (CYP3A4, CYP7A1, CYP7B1, CYP8B1, CYP27A1, CYP39A1, CYP51A1) in HepG2 and Caco2 cells following treatment with cholesterol.

Key findings: Our results showed differential influx and efflux transporters and CYP enzymes expression in cell culture models from different organs, liver (HepG2) and intestine (Caco-2). Differences in influx and efflux transporters and CYP enzymes expression between cell models of human liver and intestine origin were also observed. Conclusions: Alterations in the functional characteristics and/or expression levels of drug transporters in the liver and intestine could contribute to the intra- and interindividual variability in the oral bioavailability of drugs often observed in the clinical situations.

Keywords: cholesterol, uptake transporter, Cytochrome P450, ABC transporter, Caco-2 cells, HepG2 cells

Abbreviations: CYP, cytochrome P450; NTCP, sodium taurocholate co-transporting polypeptide; OATP1A2, organic anion transporting polypeptide 1A2; OCT1, organic cation transporter 1; LXR, liver X receptor; FXR, farnesoid X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; ROR, retinoic acid receptor-related orphan receptor; SHP, short heterodimer partner; HNF1alpha, hepatocyte nuclear factor 1-alpha; PXR, pregnane X receptor; CAR, constitutive androstane receptor; STAT5, signal transducer and activator of transcription 5.

INTRODUCTION

Cardiovascular diseases are the major causes of death and morbidity in developed countries and result in a huge financial burden on the economy. Although several risk factors have been associated to cardiovascular diseases, the molecular mechanisms involved in the regulation of homeostasis are not completely characterized. Therefore, during the past decade there was an increased awareness of the critical interplay between drug metabolizing enzymes and transporters as determinants of drug disposition and response. Several drug metabolizing enzymes and transporters are able to act on both endogenous and exogenous substrates, constituting a potential focus of interface between specific physiological and pathological states and the clearance of xenobiotic substrates such as therapeutic drugs.

Alterations in the functional characteristics and/or expression levels of drug transporters and drug metabolizing enzymes in the liver and intestine may conceivably contribute to the variability in drug oral bioavailability often observed in the clinical settings.

The liver is the major detoxification organ, which extracts drugs and endogenous compounds from blood via SLC (Solute Carrier) transporters to be metabolized by phase I and II enzymes. Metabolic products are then excreted from cell through the action of a number of ATP-dependent membrane transport proteins.

Some influx transporters are highly expressed in the liver, in which a key role in the disposition and efficacy of many drugs, such as statins, has been established [1-3]. In fact, polymorphisms of SLCO1B1 and SLCO1B3 were associated with altered transport activity of pravastatin [4] and paclitaxel [5], respectively. Hence pharmacodynamic and pharmacokinetic drug interactions can be responsible for interindividual variation in response to statins, and these uptake transporters might play a role in drug interactions.

Cytochromes P450 are involved in the metabolism of several endogenous compounds giving origin to products that elicit physiological responses [6, 7]. CYP isoenzymes participate in the mechanisms that control cholesterol homeostasis, catalyzing key steps in cholesterol metabolism, either in the synthesis or in the elimination through conversion to metabolites such as bile acids, being these substrates for CYP-mediated metabolism [8]. Besides the role of CYPs in the homeostasis of endogenous substrates, these enzymes may be of crucial importance in the metabolism of drugs used to treat cardiovascular diseases [9].

To avoid intracellular accumulation of metabolites, an active transport is mediated by ABC (ATP-binding cassette) transporters, which control the absorption and elimination. ABC transporters have been implicated in bile acid, phospholipids and sterol transport, in which the expression of these transporters is itself controlled by lipids [10].

Cholesterol plays an essential role in mammalian cells and its homeostasis is maintained by a delicate balance between cholesterol input and output pathways. Bile acids are important regulators of cholesterol homeostasis by inhibiting hepatic cholesterol metabolism into bile acids or by enhancing uptake of dietary cholesterol. Thus, the levels of bile acids and cholesterol are linked and tightly controlled [11]. The control of cholesterol homeostasis occurs essentially at

transcriptional level, and the nuclear receptors, such as farnesoid X receptor (FXR), liver X receptor (LXR), pregnane X receptor (PXR), constitutive androstane receptor (CAR) and retinoic acid receptor-related orphan receptor (ROR), play key roles in the regulation of lipid metabolism [12-15].

The mechanisms by which cholesterol regulates its conversion to bile acids remain unclear. Bile acids can bind the nuclear receptors FXR, LXR and VDR, which play important roles in lipid metabolism, as well as in detoxification of several drugs [15]. Although LXR has an important role in CYP enzymes regulation, PXR is the master regulator of these drug metabolizing enzymes, affecting cholesterol metabolism. Also CAR has an important role in the regulation of CYP enzymes and cholesterol homeostasis [16]. PXR induces CYP3A4 expression as other CYP enzymes and can be activated by several compounds such as some statins and bile acids [17]. It was found a PXR agonist that up-regulates CYP27A1 in the intestine, suggesting a LXRα-mediated activation of cholesterol efflux from intestinal cells to apoAI and HDL [18]. CYP3A4 is involved in the conversion of cholesterol to 4β-hydroxycholesterol [19] and is a key enzyme in the metabolism of a broad range of drugs, affecting their pharmacokinetic and pharmacological activities [20]. Although CYP3A4 does not play the key role in cholesterol catabolism it may be important in transcriptional regulation of lipid metabolism.

The effects of cholesterol itself on the expression of genes involved in the metabolism and transport of endogenous and exogenous compounds are however poorly understood. It is conceivable that changes in transporter and drug metabolizing enzymes expression due to altered levels of endogenous signals, such as cholesterol or bile acids, may lead to altered function of the drug metabolism/transport system, therefore affecting drug efficacy or safety.

In the present study we examined, in human hepatocarcinoma HepG2 and colon adenocarcinoma Caco-2 cells, the effect of cholesterol on the expression of selected human genes involved in the metabolism and transport of endogenous and exogenous compounds: SLC10A1, SLC22A1, SLC22A7, SLC01A2, SLCO1B1, SLCO1B3, SLCO2B1, ABCA1, ABCB11, ABCC2, ABCC3, ABCC6, ABCG2, ABCG5, ABCG8, CYP3A4, CYP7A1, CYP7B1, CYP8B1, CYP27A1, CYP39A1 and CYP51A1.

The aim of this work is to provide a fundamental resource to the understanding of the molecular mechanisms underlying the susceptibility of a given individual to disorders in cholesterol and bile acids homeostasis. This knowledge should provide important clues for future improved surveillance in the field of cardiovascular diseases.

MATERIALS AND METHODS

Cell Culture. The human hepatoma cell line HepG2 and the human colon adenocarcinoma Caco-2 were maintained in Dulbecco's modified medium (DMEM) supplemented with 10% and 20% fetal bovine serum, respectively, 1% glutamine penicillin streptomycin, and 1% non-essential amino acids at 37ºC in a humidified 5% CO_2, 95% air atmosphere.

Cell Treatments. Before treatment, cells were sequentially adapted to an almost serum-free environment. HepG2 cells were adapted to 0,05% FBS and Caco-2 cells to 1% FBS. The same treatment was made to Caco-2 20% FBS. HepG2 and Caco-2 cells were treated with 1-20µg/ml cholesterol (Sigma-Aldrich). Ethanol was used as control vehicle. Treatment times were 0, 2, 4, 8, 12 and 24 hours.

RNA Isolation. Total RNA was isolated from HepG2 and Caco-2 cells using a modification of the guanidine tiocyanate method [21]. The RNA was then purified from contaminating genomic DNA by incubation with DNase I (3 U/100µL RNA, Promega) at 37ºC for 30 min, followed by heat inactivation of the enzyme at 55ºC for 10 min.

Primers. Gene-specific primers were used for RT-PCR amplification as indicated in table I. Primers for human ribosomal RNA 18S were used as a positive control for efficiency of RNA isolation and cDNA synthesis.

Table 1: Specific primer sequences used in RT-PCR analysis

Gene	Accession number	Forward primer (5'→3')	Reverse Primer (5'→3')
18S	NC_000024.8	TGAAACTGCGAATGGCTCAT	CGACTACCATCGAAAGTTGA
SLC10A1	NC_000014.7	GGGACATGAACCTCAGCATT	CGTTTGGATTTGAGGACGAT
SLC22A1	NC_000006.10	TAATGGACCACATCGCTCAA	AGCCCCTGATAGAGCACAGA
SLC22A7	NC_000006.10	GTGGTGCTAGGACTGCCAAT	ATGAGACCAGTGGGTTGGAG
SLCO1A2	NC_000012.10	TGGGGAACTTTGAAATGTGG	AAGGCTGGAACAAAGCTTGA
SLCO1B1	NC_000012.10	ACTGATTCTCGATGGGTTGG	TTTCCAGCACATGCAAAGAC
SLCO1B3	NC_000012.10	TCCCTCTAATCTGCGAAAGC	CACAGACTGGTTCCCACTG
SLCO2B1	NC_000011.8	TACCGCTACGACAACACCAG	GATCCCCACCACACTCAGAT
CYP3A4	NC_000007.12	GGATCCATTCTTTCTCTCAAT	GTATCTTCGAGGCGACTTTC
CYP7A1	NC_000008.9	AATGTCCACCTTTGGACCAG	AGGGTGTTCTGCAGTCCTGT
CYP27A1	NC_000002.10	GCAGAGACAGTGCTGAGCTG	AGGGTGTGTGCAAGGAGTTC
CYP39A1	NC_000006.10	TTTCTGCTTCTGGAAGGTGCTGGA	ACAGAGCAAGGCAACCCAGGATTA
CYP51A1	NC_000007.12	TTTGCCTAGTTTCAGACGCAGGGA	TCATCATCAGTCAAAGGACGCCCA
CYP7B1	NC_000008.9	AAGCAGGCAAGATGTCCTGGAGAA	AACGGTCAATTTCGTCACGCACTG
CYP8B1	NC_000003.10	AACAGTGTGCGTGTCGAGAGCTTA	TATCCAGCAATGACCACCAGCAGA
ABCA1	NC_000009.10	TGGCTTAGATTGGACAGCCCAAGA	AGCCAGACTTCTGTTGCTATGGGT
ABCB11	NC_000002.10	ATGGACCTGCCACAGCAATTTGAC	ATCTTGGGATTTCGGATGAGGGCT
ABCC2	NC_000010.9	TGAGCAAGTTTGAAACGCACAT	AGCTCTTCTCCTGCCGTCTCT
ABCC3	NC_000017.9	CTATGTGCCCCAGCAGGCATG	CCAATCTCTGTCTGATCCCCACCAGGCA
ABCC6	NC_000016.8	CTCTACGCTGGGTTTCAGAGCCTG	CTACAGCCATGTGGGAGCAGACAG
ABCG2	NC_000004.10	GGTGGTACAAGATGATGTTGTGAT	GCCGAAGAGCTGCTGAGAAC
ABCG5	NC_000002.10	ACCGAATTGTGGTTCTCACC	AAGGGTAACCGCAGTCATTG
ABCG8	NC_000002.10	ACGCCATCTACCTCATCGTC	TTGAAGGGTCTGCTCAGGTC

Reverse Transcriptase Polymerase Chain Reaction. RNA (400 ng) was transcribed by reverse transcriptase (50 U, Invitrogen) for 50 min at 37ºC. The reverse transcriptase was inactivated by heating to 70ºC for 15 min. PCR was performed in the presence of 1-4mM $MgCl_2$, 1x PCR buffer (Fermentas), 0.2mM dNTPs, 2-4pmol/µL of forward and reverse primers and 1.5U Taq polymerase (Fermentas). PCR amplifications were conducted using one denaturation step at 94ºC for 2 min, followed by 40-45 cycles of 92.5ºC for 30 s, 40-55ºC for 45 s and 72ºC for 1 min and final PCR extension of 3 min at 72ºC (Eppendorf Mastercycler). Relative quantification of amplification products was performed by densitometric analysis (AlphaInnotech).

Statistical Analysis. Results are expressed as mean ± SD of three assays per condition, after normalization with the levels determined for the 18s rRNA.

RESULTS

We aimed to evaluate the effect of cholesterol on the expression of solute carriers SLC10A1 (NTCP), SLC22A1 (OCT-1), SLC22A7 (OAT2), SLCO1A2 (OATP-A), SLCO1B1 (OATP-C), SLCO1B3 (OATP-8) and SLCO2B1 (OATP-B), CYP enzymes CYP3A4, CYP7A1, CYP7B1, CYP8B1, CYP27A1, CYP39A1, CYP51A1 and ABC transporters ABCA1, ABCB11 (BSEP), ABCC2 (MRP2), ABCC3 (MRP3), ABCC6 (MRP6), ABCG2 (BCRP), ABCG5 and ABCG8 in human HepG2 and Caco-2 cells.

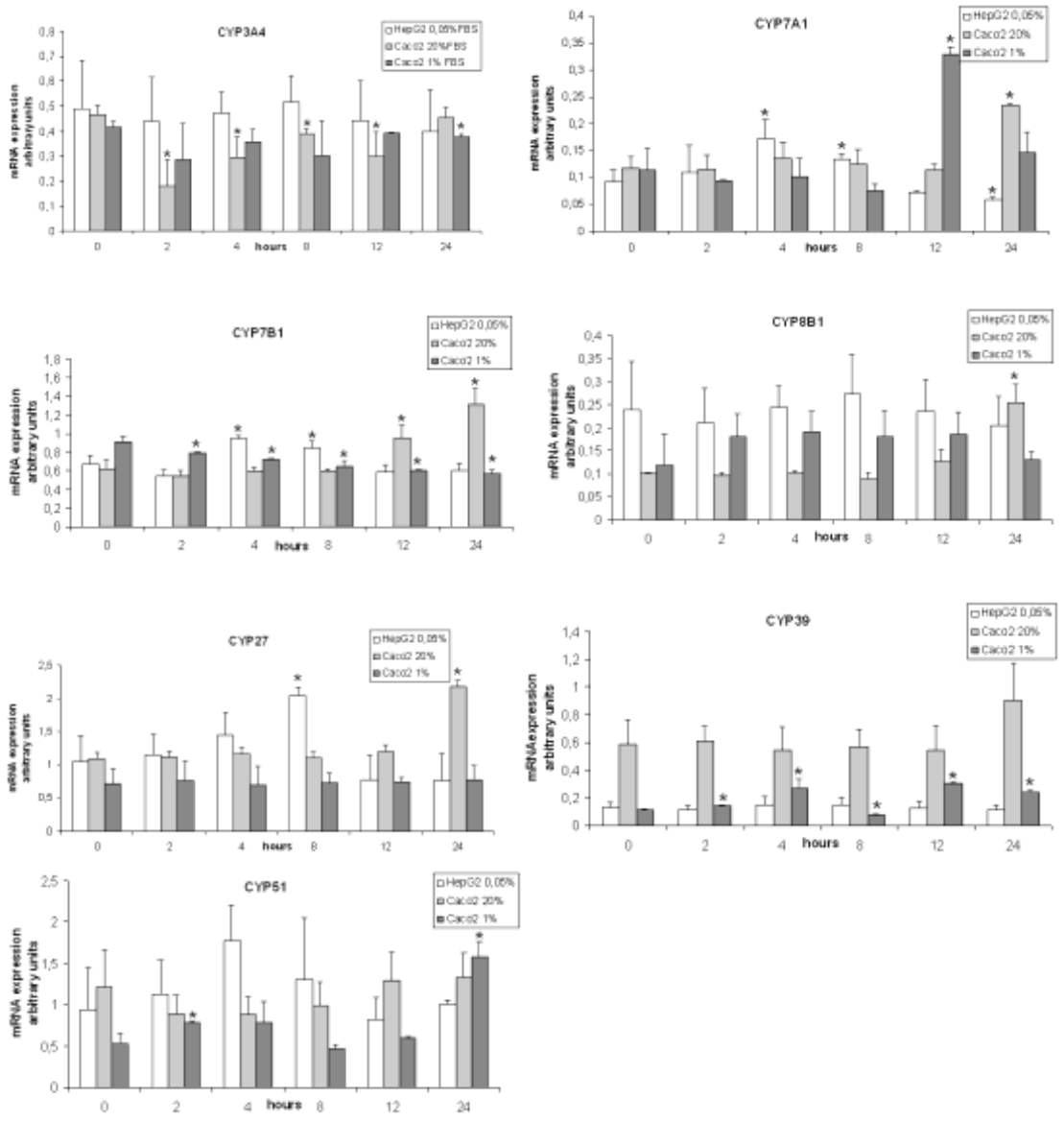

Figure 1: Effect of cholesterol on the expression of CYP enzymes in HepG2 and Caco2 cells. Cell culture media was supplemented with 5µg/ml cholesterol for different periods as indicated. CYPs mRNA relative levels were assessed by RT-PCR analysis using specific primers as described in Table 1. Results are expressed as relative mRNA expression normalized to internal control 18S mRNA. Error bars represent the standard deviation from the mean of triplicate assays of an individual experiment.

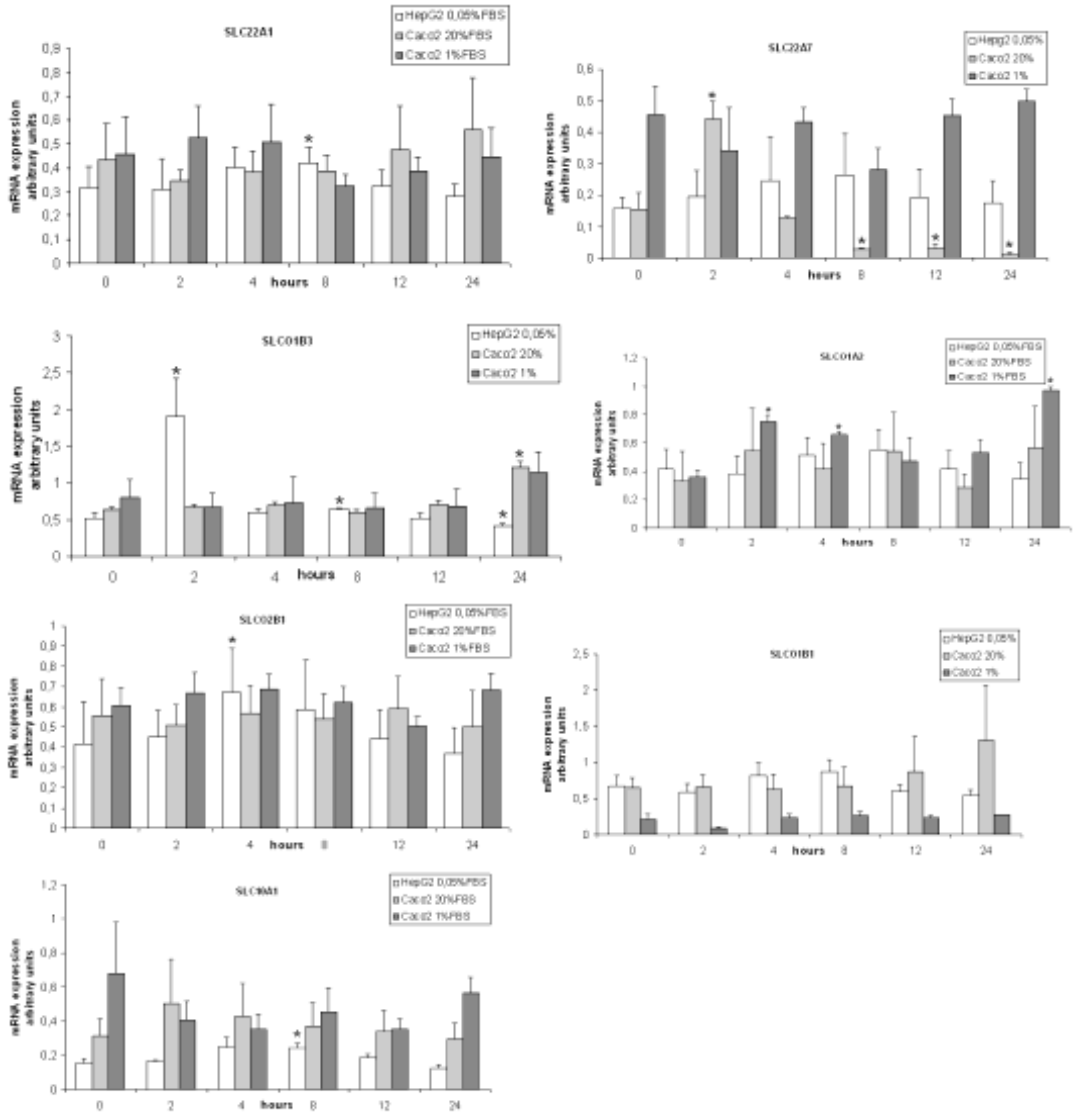

Figure 2: Effect of cholesterol on the expression of influx drug transporters in HepG2 and Caco2 cells. Cells were treated with 5µg/ml cholesterol for different periods as indicated. mRNA relative levels for influx transporters were evaluated by RT-PCR analysis using specific primers as described in Table 1. Results are expressed as relative mRNA levels normalized to the internal control 18S mRNA. Error bars represent the standard deviation from the mean of triplicate assays of an individual experiment.

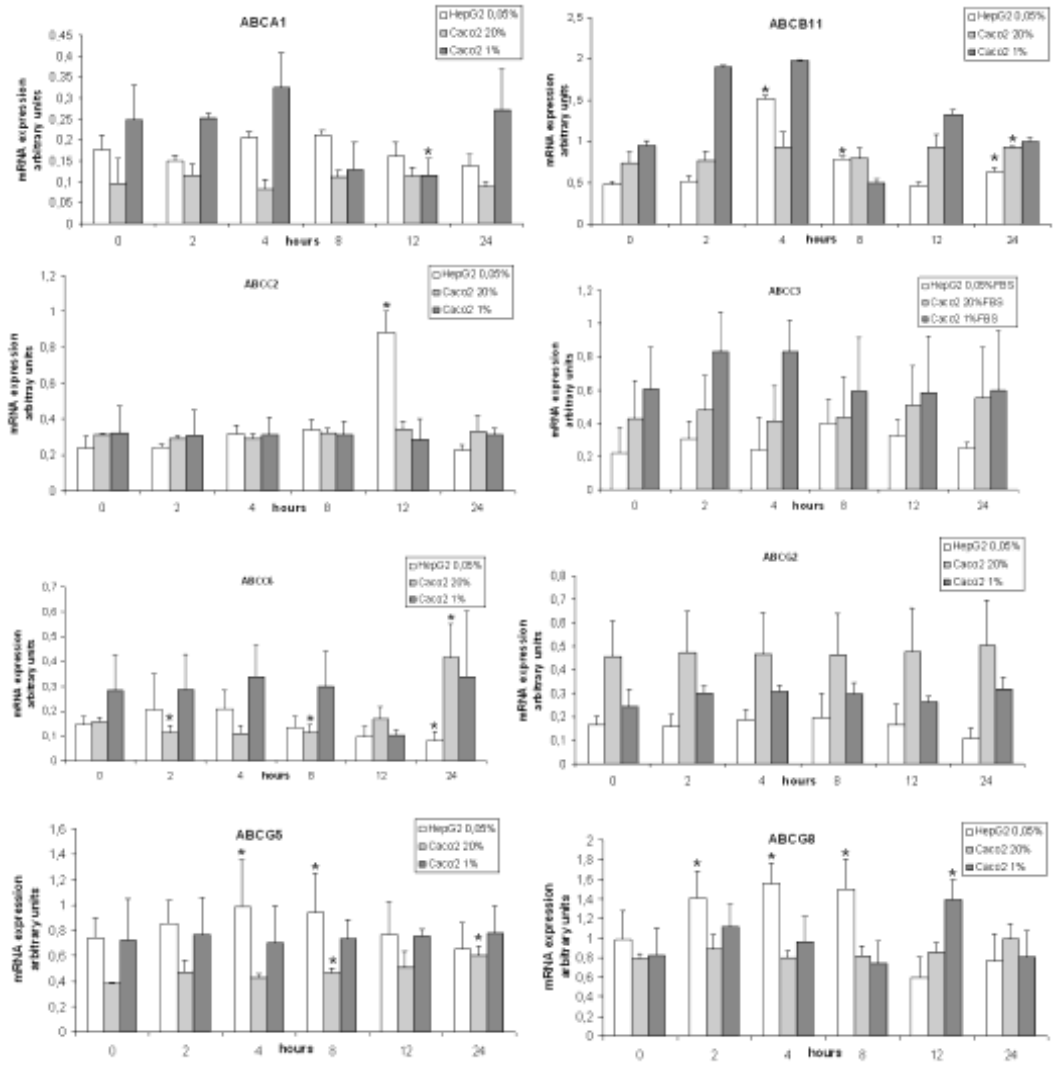

Figure 3: Effect of cholesterol on the expression of ABC transporters in HepG2 and Caco2 cells.
Cells were treated with 5µg/ml cholesterol for different periods as indicated. mRNA relative levels for ABC transporters were evaluated by RT-PCR analysis using specific primers as described in Table 1. Results are expressed as relative mRNA levels normalized to the internal control 18S mRNA. Error bars represent the standard deviation from the mean of triplicate assays of an individual experiment.

Different cholesterol concentrations were tested in this study, ranging from 1 to 20µg/ml, for different treatment periods from 2 to 24h. mRNA levels of CYP enzymes and influx and efflux transporters were detected in Caco-2 and HepG2 cells in different patterns of expression (Figures 1, 2 and 3).

No significant changes were observed in the relative levels of SLCO1B1, ABCC3 and ABCG2 mRNA in Caco-2 and HepG2 cells after cholesterol treatment (Figures 1 and 3).

For HepG2 cells, significant alterations in the expression levels occurred mainly after 4 or 8h of treatment and were observed to SLCO1B3, SLC10A1, SLC22A1, CYP7A1, CYP27A1, CYP7B1, ABCB11, ABCG5 and ABCG8 (Figures 1, 2 and 3).

In Caco-2 cells, we observed differences in gene expression if the cells were adapted to a serum-free medium (1% FBS) or if they were in a serum-rich medium (20% FBS). Caco-2 cells with higher serum concentration (20%) were more responsive to cholesterol than Caco-2 cells

with low serum concentration (1%). However significant alterations in the expression levels occurred at longer periods of treatment (12 and 24h) than those observed for HepG2 cells (Figures 1, 2 and 3). In Caco-2 cells with 20% serum the mRNA expression was altered in SLCO1B3, SLC22A7, CYP3A4, CYP7A1, CYP27A1, CYP7B1, CYP8B1, ABCB11, ABCC2, ABCC6 and ABCG5. In Caco-2 cells with 1% serum the mRNA expression was altered in SLCO1A2, CYP7A1, CYP39A1, CYP51A1, CYP7B1, ABCA1 and ABCG8.

The expression pattern observed for all concentrations tested was not significantly altered and the induction effect, when observed was maximal for the concentration 5µg/mL.

All the genes analysed were detected in untreated HepG2 and Caco-2 cells, in accordance with previous reports [22-29].

Different gene expression pattern was observed for HepG2 and Caco-2 cells. Genes that were highly expressed in both cell lines included SLC22A7 (OAT2), CYP51A1, ABCC2 (MRP2), ABCC3 (MRP3) and ABCG2 (BCRP), whereas in Hep2 cells SLC10A1 (NTCP), ABCG5 and ABCG8 were also high expressed and in Caco-2 SLCO2B1 (OATP-B), CYP3A4, ABCA1 and ABCC6 (MRP6). Genes moderately expressed in both cell lines were CYP27A1 and CYP8B1, in HepG2 cells were SLCO2B1 (OATP-B), SLC22A1 (OCT1), CYP3A4, CYP7B1, ABCA1 and ABCC6 (MRP6) and in Caco-2 cells were SLCO1A2 (OATP-A), SLC10A1 (NTCP), CYP39A1 and ABCB11 (BSEP). Genes expressed at a low level at both cell lines included SLCO1B1 (OATP-C), SLCO1B3 (OATP-8) and CYP7A1, in HepG2 were SLCO1A2 (OATP-A), CYP39A1 and ABCB11 (BSEP) and in Caco-2 SLC22A1 (OCT1), CYP7B1, ABCG5 and ABCG8.

Our results showed differential influx and efflux transporters and CYP enzymes expression in cell culture models from different organs, liver (HepG2) and intestine (Caco-2). Differences in influx and efflux transporters and CYP enzymes expression between cell models of human liver and intestine origin were also observed, indicating that these genes are differently regulated in these organs.

DISCUSSION

Influx and efflux transporters among with metabolizing enzymes are able to act on endogenous and exogenous substrates, being involved in specific physiological and pathological states and in the clearance of xenobiotic substrates such as therapeutic drugs. Therefore, the interplay between transporters and drug metabolizing enzymes is determinant for drug disposition and response. In this context, the effects of cholesterol on drug metabolizing enzymes and transporters should provide valuable information to the development of novel, more effective and safe therapeutics.

Influx transporters

While human SLCO1B3 is up-regulated by bile acids, SLC10A1, SLC22A1 and SLC22A7 are down-regulated by bile acids through the action of FXR [30-32]. This action is mediated by a small heterodimer partner (SHP)-1 and inhibits the function of hepatocyte nuclear factor (HNF)-1α [33].

A decrease in SLC10A1 expression was reported in percutaneous liver biopsy samples of patients with cholestatic liver disease [34] and in rat liver, as the uptake of bile salts is markedly impaired in cholestasis [35]. However, in HepG2 cells the levels of SLC10A1 increased in response to cholesterol, indicating a positive regulation of this gene [36].

Cholesterol is converted to bile acids, which are uptake by liver cells by two mechanisms, one Na^+-dependent due to NTCP and another Na^+-independent due to OATP family [37]. OATP-C and OATP-8 have high homology and broad substrate specificity, but OATP-8 is highly expressed in cancer cells [38], which can explain the differences observed in the expression pattern. Variants of SLCO1B1 have been associated with changes in cholesterol synthesis rate [39] and with lipid-lowering dugs efficacy [40, 41].

mRNA levels of SLCO1A2 were reduced in rat liver in ethinylestradiol-induced cholestasis, associated with decreased binding of PXR [35], which is in agreement with reports from PXR null mice, suggesting a coordinated role of PXR in the regulation of both SLCO1A2 and CYP7A1 [42]. HNF-1α is also involved in transcriptional regulation of SLCO1A2 [43].

OATP-B is ubiquitously expressed but has high substrate specificity. The transcription factor Sp1 was found to be necessary for constitutive expression of this gene in liver and intestine and SLCO2B1 was found to be repressed by CAR and AhR activator in human hepatocytes, which play a key role in the regulation of lipid and bile acids homeostasis [16, 44].

The mRNA and protein levels of SLC22A1 were described as down-regulated in bile duct ligation in the rat, a model of obstructive cholestasis but, in kidney the opposite was observed with the up-regulation of SLC22A1 [46].

Recently was described by Rodrigues *et al* that the levels of SLC22A1 and SLCO2B1 mRNA are increased in patients with hypercholesterolemia, positively correlated with LDL cholesterol and total serum concentrations and, after atorvastatin therapy SLC22A1 gene expression was down-regulated [47]. Besides SLC22A1 and SLCO2B1, in Caco2 cells were significantly down-

regulated after treatment with simvastatin while in HepG2 cells SLCO2B1 was up-regulated by atorvastatin [27]. These results are in agreement with the induction observed for these two genes in HepG2 after cholesterol treatment [36].

SLC22A7 is activated by HNF-4α, which has as targets, genes that encode proteins involved in cholesterol metabolism. mRNA levels of this transporter were down-regulated after treatment with bile acids in Huh7 cells [32] as well in obstructive cholestasis in rats [46]. Bile acids are strong regulators of FXR, which induces the transcription of its target genes via SHP [48].

Drug metabolizing enzymes

In primary hepatocytes, bile acids down-regulate CYP7A1 and its synthesis by activation of the JNK1/2 signaling pathway [49, 50]. However, in a model of bile duct ligation in the rat, bile acids induced rat CYP7A1 transcription [51]. Cholesterol and oxysterols have a controversial role in CYP7A1 regulation since a wide species-related variability has been reported [52-55].

CYP7B1 is expressed in liver and steroidogenesis tissues and it has high homology with CYP7A1 [56] being hypothesized as the main function the inactivation of oxysterols, protecting the liver from cholestasis [57]. Ren *et al*, described that the CYP7B1 mRNA expression was increased in primary rat hepatocytes treated with cholesterol, suggesting that cholesterol might regulate CYP7B1 [58]. These results are in agreement with our observations in HepG2 cells.

CYP8B1 is expressed in liver where it is an obligatory enzyme in the synthesis of cholic acid [59]. CYP8B1 is up-regulated by a bile acid-binding resin and down-regulated by bile acid ingestion such as CYP7A1, but to a lesser extent [60]. Studies in rats demonstrated that cholesterol, thyroid hormones and insulin regulate CYP8B1 expression at transcriptional level by decreasing CYP8B1 mRNA levels, while starvation has the opposite effect [61].

CYP7A1, CYP7B1, CYP8B1 and CYP27A1 in the presence of high levels of bile acids are repressed via FXR or PXR by complex mechanisms [14], which involve the induction of SHP in liver or the induction of FGF19 to activate liver FGF receptor 4 signaling in the intestine. Bile acids also bind PXR, VDR or CAR which inhibit gene transcription via interactions with HNF4α [62-64].

CYP27A1 is a ubiquitous enzyme that catalyzes the 27-hydroxylation of cholesterol and this pathway is strongly up-regulated when the classical pathway is suppressed, indicating a potential regulatory role for CYP27A1 [19]. Bile acids also down-regulate CYP27A1 mRNA levels and enzyme activity, however with a smaller effect than the observed for CYP7A1 [65]. Accordingly with Li *et al*, cholesterol up-regulates CYP27A1 expression in intestine but not in liver cells [50], which is in agreement with our results.

In cholestatic patients occurs an increased excretion of the marker of CYP3A4 activity, 6β-hydroxylated bile acid, indicating that this enzyme is induced in response to cholestasis [66]. FXR also regulates the expression of CYP3A4, inducing this gene, which in turn oxidizes bile acids detoxifying them [67].

CYP39A1 is abundantly and constitutively expressed in liver [68]. Little is known about the regulation of CYP39A1 but, in mice the mRNA levels of this enzyme remained unaltered in response to dietary cholesterol, bile acids and a bile-acid binding resin [19].
CYP51A1 is a ubiquitous enzyme involved in cholesterol biosynthesis and has a regulatory role acting as down-regulator of cholesterol biosynthesis by suppression of HMG-CoA reductase translation [69]. The regulation of CYP51A1 occurs mainly at transcriptional level with low levels of cholesterol leading to the increase of CYP51A1 mRNA levels[19].

ABC transporters

Besides solute carriers and drug metabolizing enzymes, nuclear receptors are also regulators of ABC transporters. In fact, LXR induces transcription of ABCA1, ABCG5 and ABCG8, which play key roles in cholesterol homeostasis [67].
Controversy results about regulation of ABCA1, in macrophages, by statins have been published [70, 71]. However, changes in cholesterol levels and macrophage differentiation, seems to affect ABCA1 gene expression in response to statins, possibly affecting oxysterols availability [72].
ABCB11 is involved in the transport of bile salts from the liver to the bile and is positively regulated by FXR [73, 74]. In cholestatic liver disease the levels of bile acids are increased and the regulation by FXR/RXR plays a key role in the protective mechanism of hepatocytes [75].
ABCC6 is expressed mainly in liver and kidney, but also in intestine [76].
ABCC3 is expressed on the basolateral membrane in liver and intestine and overlaps substrate specificity with ABCC2, such as for bile acids [77]. Several liver disorders, such as obstructive cholestasis result in increased ABCC3 protein levels [78] and it was reported that PXR and CAR up-regulate ABCC3 expression [79]. Besides, LRH-1 up-regulates ABCC3, a response mediated by bile acids [80].
ABCC2 is expressed at the apical membrane in liver and intestine and plays a functional role in the intestinal secretion of many drugs [81]. It was reported that ABCC2-deficient and bile duct ligation rats had hepatic ABCC3 mRNA expression enhanced [82]. The nuclear receptors FXR, CAR and PXR are regulators of ABCC2 expression in human and rat hepatocytes [79, 83].
Activity of ABCG2 is inhibited by membrane cholesterol depletion [84]. Aryl hydrocarbon receptor (AhR), nuclear factor E2-related factor (Nrf2) and PXR were associated with ABCG2 induction [44].
Rodrigues *et al*, described that the levels of ABCC2 and ABCG2 mRNA are increased in patients with hypercholesterolemia, positively correlated with LDL cholesterol and total serum concentrations and, after atorvastatin therapy gene expression was down-regulated [47]. In Caco2 cells, ABCC2 was significantly down-regulated after treatment with simvastatin while in HepG2 cells SLCO2B1 was up-regulated by atorvastatin [27]. The increase of ABCC2 in hypercholesterolemia is in agreement with our results, in HepG2 cells.

ABCG5 and ABCG8 are involved in the absorption of cholesterol from diet and in the secretion of cholesterol from bile [67]. ABCG5 and ABCG8 are expressed in liver and intestine and need to dimerize with each other to become active membrane transporters. These transporters are up-regulated through LXR/RXR [85]. In hamster or mice, the addition of cholesterol to the diet resulted in an increase of ABCG5 and ABCG8 in liver and small intestine [85, 86], which is in agreement with our results.

The expression of mRNA levels has been compared between Caco-2 cells and human intestine and, some authors have observed that Caco-2 expression pattern was clearly distinguishable from that found in human small intestine [25] but was in good agreement between human jejunum [22].

CONCLUSION

The results obtained in this work point, in general, to an increased expression of uptake and efflux transporters as well as drug metabolizing enzymes triggered by cholesterol. As the majority of the studied genes is known to be repressed by bile acids indicating that in rat liver, almost all cholesterol is metabolized to bile acids, which at high levels in the cell can repress target genes, in HepG2 cells the metabolism of cholesterol may be reduced and bile acids should be present at low levels. Cholesterol mediated changes might occur at transcriptional level and the metabolites of cholesterol or even cholesterol by itself may activate nuclear receptors that regulate the expression of uptake and efflux transporters and drug metabolizing enzymes. Oxysterols are bile acids precursors and they can bind nuclear receptors, such as FXR, LXR, PXR and CAR activating regulatory mechanisms of cellular bile acids balance.

Our hypothesis is that the several metabolic enzymes and transporters may work in a coordinated fashion to control homeostasis of endogenous compounds relevant for the development of cardiovascular pathologies. The changes observed in the levels of drug metabolizing enzymes and transporters in response to cholesterol would affect therapeutic success.

Our results should contribute to a better understanding of the molecular mechanisms underlying the susceptibility of a given individual to disorders in cholesterol and bile acid homeostasis, as well as to high blood pressure. This knowledge should provide important clues for future improved surveillance in the field of cardiovascular diseases.

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Chapter IV - Discussion

In the present work we focused on the pharmacogenetics as well as on the expression profiles of genes that are candidate to play key roles in susceptibility and treatment of cardiovascular pathologies. Several metabolic enzymes and transporters are involved in the control of homeostasis of endogenous compounds, such as cholesterol, a known risk factor for the development of cardiovascular diseases. On the other hand, if changes in the levels of endogenous compounds are found to regulate expression of drug metabolizing enzymes and transporters, disease status itself will affect therapeutic success. The results obtained demonstrate a clear pattern of geographical distribution of pharmacogenetically relevant polymorphisms, which combined with altered expression profiles of the respective genes, may have a crucial relevance for the development, progression and therapeutics of cholesterol related diseases.

1. Pharmacogenetics of genes candidate to play a role in cardiovascular disease risk and therapeutics

Drug metabolizing enzymes and transporters show a high degree of variability, which determines the final circulating levels of the substrates in each individual. This reflects the existence of genetic polymorphisms which may determine the individual risk for a given pathology, and/or the efficacy of the therapeutic strategy.
The main drugs used in the treatment of cardiovascular diseases are statins, which act as HMG-CoA reductase inhibitors. The most widely used statins - simvastatin, rosuvastatin and pravastatin - are well tolerated and appear to have a wide safety margin (Franke *et al*, 2010). Although statins have relatively low-to-medium bioavailability, they are associated with high inter-individual pharmacokinetic variability. Statins are primarily metabolized in the liver and so, genetic variation in the genes involved in the transport and metabolism of these compounds can give important clues for this variability. Uptake transporters, particularly belonging to the OATP family, are responsible for the hepatic uptake and are the main candidates for inter-individual pharmacokinetic variability since this step could be a critically important rate-limiting step in their clearance. Therefore, the efficacy and safety of treatment depends on an optimized dosage that should be adjusted for each patient individually, according with his genetic profile.

The genes coding for influx and efflux transporters and drug metabolizing enzymes are polymorphic, an increasing number of SNPs having emerged recently in the literature, most of them localized in the coding region, with a potential impact on the transporter structure and/or activity.
In the course of this study we developed methods to identify relevant variants in transporter-coding genes and characterized their prevalence in a sample of the Portuguese population.

Next we compared the prevalence of these SNPs in Portuguese with the prevalence in individuals from distinct/geographic background, from Mozambique and Colombia.

PCR-RFLP genotyping methods were developed for SNPs in genes coding for metabolizing enzymes (*CYP7A1-* A-203C, C-278A, C-496T, A698G, G1039A; *CYP7B1-* C-116G, C-1774T, G971A; *CYP27A1-* C490T, C506T, G817A, T1633C), and for influx (*SLCO1B1-* T217C, A388G, C463A, T521C; *SLCO1B3-* T334G, G699A, G1564T; *SLC22A1-* C480G, C886G, T4215C; *SLC10A1-* C800T, A2587G, A2192G) and efflux (*ABCB11-* T580C, G779A, C1331T, A2155G; *ABCG5-* G80C, C148T, C1550G, C1810G; *ABCG8-* G55C, A161G, C1199A, T1895C) transporters.

Influx transporters

In humans, OATP-C (*OATP1B1*) and OATP-8 (*OATP1B3*) have high homology, being both predominantly expressed on the basolateral membrane of hepatocytes (Konig *et al*, 2000a). Their main function is the removal of substrates from blood into the liver, displaying a certain degree of overlap in substrate specificity, since both transporters are involved in the uptake of similar endogenous substrates, such as bilirrubin, bile acids, conjugated steroids, eicosanoids and thyroid hormones and various drugs, including the endothelin receptor antagonist BQ123, the antibiotic rifampicin and some statins, such as pravastatin and pitavastatin, indicating a potential role in the modulation of lipid-lowering therapy (Kalliokoski and Niemi, 2009; Konig *et al*, 2006; Hsiang *et al*, 1999; Nozawa *et al*, 2004). However, OATP-8 appears to be unique in digoxin transport and possibly also in the docetaxel and paclitaxel transport, indicating an important role in the treatment of congestive heart failure and cancer (Kullak-Ublick et al, 2001; Smith *et al*, 2007).

In the case of the *SLCO1B1* gene, the variants A388G and T521C were described as being in linkage disequilibrium in patient populations (Franke *et al*, 2010), although this correlation was not observed for healthy individuals from European or African origin (Mwinyi *et al*, 2008). Consequently, six haplotypes have been described in patient populations, *SLCO1B1**1A (-11187G/-10499A/388A/521T), *SLCO1B1**1B (-11187G/-10499A/388G/521T), *SLCO1B1**5 (-11187G/-10499A/388A/521C), *SLCO1B1**15 (-11187G/-10499A/388G/521C), *SLCO1B1**16 (-11187A/-10499A/388G/521C) and *SLCO1B1**17 (-11187A/-10499A/388G/521C), the last two haplotypes being specific of patients of European ancestry (Franke *et al*, 2010).

Here, we analysed *SLCO1B1* T217C (F73L), A388G (N130D), C463A (P155T) and T521C (V174A) polymorphisms (Results - Paper I). From these, only T217C was not detected in any individual analysed from Portugal, Mozambique or Colombia, which is in agreement with Mwinyi *et al*, in a study for for a German population (Mwinyi *et al*, 2008). Tirona *et al*, also described this variant as absent in African Americans but as rare (2%) for an European American

population (Tirona *et al*, 2001). This polymorphism results in the amino acid exchange Phe73Leu (F73L), which is highly conserved among rat and mouse orthologs of SLCO1B1. Besides, this polymorphism is localized in the putative transmembrane-spanning domain and was associated with a significant reduction in transport activity and a decreased fractional cell membrane expression (Tirona *et al*, 2001). In this way, the absence of this polymorphism, in these three analysed populations, may indicate that this region is of fundamental importance for the protein anchorage to the membrane, which may also explain the reduction in transport activity.

For the *SLCO1B1* C463A (P155T) polymorphism, the variant allele was detected at a high frequency among Portuguese (23.4%) and Mozambican (16.6%), significantly higher than in Colombians (5.5%). Although our results for the Portuguese population were slightly higher than previous results reported for Germans (16%), Finnish (13.1%) and European Americans (16.3%), the difference does not reach statistical significance (Mwinyi *et al*, 2008; Pasanen *et al*, 2006; Tirona *et al*, 2001). However, the same doesn't happen when comparing the allelic frequencies obtained in Mozambique with another African population from Uganda (2.2%) or with an African American population (2.3%), which may be explained by the different ethnic groups that exist in Mozambique (African 99.66% -Makhuwa, Tsonga, Lomwe, Sena, and others, Europeans 0.06% - Euro-Africans 0.2%, Indians 0.08%) and Uganda (Baganda 16.9%, Banyakole 9.5%, Basoga 8.4%, Bakiga 6.9%, Iteso 6.4%, Langi 6.1%, Acholi 4.7%, Bagisu 4.6%, Lugbara 4.2%, Bunyoro 2.7%, other 29.6%) that confer a great genetic diversity in the African continent (Mwinyi *et al*, 2008; Tirona *et al*, 2001). Besides, African Americans are a mixture of Africans from different geographical localizations form the African Continent, giving a wide genetic diversity to this population.

As observed for T217C, C463A was also associated with a significant reduction in transport activity (Tirona *et al*, 2001). This polymorphism is predicted to be localized on the extracellular loop 5 and this reduced activity may suggest an important role of this region to substrate-transporter interaction (Tirona *et al*, 2001). In this way, the difference observed in allelic frequencies among different populations may suggest, that some therapeutic drugs may be more or less efficacious according with the genetic profile of the population, underlying the importance of the genetic profile of the patient.

SLCO1B1 A388G (N130D) was found to be a very frequent polymorphism in Portuguese (42.4%), Mozambican (65.6%) and Colombian (47.8%) populations, the allelic frequency of the Mozambicans being statistically different from the other two populations. Our results for the Portuguese population are in agreement with studies in Finnish (46.2%), Germans (36.5%) and European Americans (30.6%) and, for the Mozambican population, with the results obtained for Uganda (77.8%) and African Americans (75%) (Mwinyi *et al*, 2008; Pasanen *et al*, 2006; Tirona *et al*, 2001). This polymorphism is localized in extracellular loop 2 and was associated only with minimal changes in the transport activity (Tirona *et al*, 2001), which may explain its high frequency. However, Pasanen *et al*, described a possible important role of this transporter in the

regulation of cholesterol concentration, since the A388G polymorphism was found to be associated with an increase of cholesterol synthesis (Pasanen *et al*, 2007).

SLCO1B1 T521C (V174A) was a frequent polymorphism in Portuguese (15.3%) and Colombian (14.8%) populations but relatively rare for the Mozambican (4%) population, being this distribution statistically significant. These results are in agreement with previous studies (Germans - 15%; Finnish – 20.2%; Uganda – 3.9%; African Americans – 2.3%) (Mwinyi *et al*, 2008; Pasanen *et al*, 2006; Tirona *et al*, 2001).

A388G and T521C were associated with a functional decrease in OATP-C activity based on altered transport of substrates, such as pravastatin, atorvastatin and cerivastatin (Kameyama *et al*, 2005; Nozawa *et al*, 2005a; Tirona *et al*, 2001; Iwai *et al*, 2004; Tirona *et a*l, 2003a) and with a reduction of cholesterol synthesis following statin administration (Hedman *et al*, 2006; Niemi *et al*, 2005a; Tachibana-Iimori *et al*, 2004).

Studies based on the systemic exposition to statins of patients with high lipid levels have demonstrated that the individuals carrying the haplotypes with the homozygous variant 521C have the highest plasma concentration of statins, suggesting that this variant leads to a decrease in function of OATP-C (Kameyama *et al*, 2005; Nozawa *et al*, 2005a; Tirona *et al*, 2001; Iwai *et al*, 2004; Tirona *et a*l, 2003a). Niemi *et al* and Pasanen *et al*, observed that the area under the curve and peak concentration of pravastatin, simvastatin, atorvastatin and rosuvastatin were significantly increased in 521C homozygous individuals (Pasanen *et al*, 2006a; Niemi *et al*, 2006; Pasanen *et al*, 2007). On the other hand, the observed decrease in the uptake of statins may have as consequence a lower inhibition of HMG-CoA reductase in the hepatocyte, making this an interesting example of a polymorphic variant that affects simultaneously the efficacy and the systemic toxicity of a therapeutic drug. Based on the differences obtained for the allelic frequencies in Portuguese, Mozambican and Colombian populations, the use of statins in therapeutics may be less effective in Portuguese and Colombian populations, since the drug remains in blood circulation and takes more time to enter in the cell to inhibit HMG-CoA reductase. These high allelic frequencies, obtained for these uptake transporters, known to be involved in the statin transport, may also help to understand why statin treatment has such high variability (Karumanchi and Thadhani, 2010).

No reports were found to compare with our results obtained in the Colombian population, highlighting the fact that, to our knowledge, this is the first report of *SLCO1B1* pharmacogenetics in a South American population.

SLCO1B3 is also a polymorphic gene and several polymorphisms have been evaluated for an effect on the pharmacokinetics of some therapeutic agents (Franke *et al*, 2010).

In this work we analysed *SLCO1B3* T334G (S112A), G699A (M233I) and G1564T (G522C) polymorphisms (Results - Paper I). For the first two variants we observed a very high allelic frequency among Portuguese (70.1% for both polymorphisms) and Colombians (T334G – 77.5%; G699A – 71.4%), but smaller for Mozambicans (T334G – 22.2% - significantly different

from Portugal and Colombia; G699A – 59.3%). The polymorphism G1564T was relatively rare among the three populations (Portugal – 5.4%; Mozambique – 3.3%; Colombia – 4.4%).

When comparing our results with others previously published we observed slight differences, but without statistical significance. *SLCO1B3* T334G (Ser112Ala) has been previously analysed in Caucasians from Central Europe (74%), European Caucasians (81%), American Caucasians (88%), African Americans (41%), Ghanaians (38%) and Mexicans (78%) by other authors (Smith *et al*, 2007; Letschert *et al*, 2004). Results for *SLCO1B3* G699A (Met233Ile) were published for Europeans (71%), American Caucasians (87%), Ghanaians (38%), African Americans (41%) and Mexicans (79%) in two different studies (Smith *et al*, 2007; Letschert *et al*, 2004).

The only report found for G1564T (Gly522Cys) was a study in an European population (1.9%) with a lower allelic frequency compared with our results (Letschert *et al*, 2004). In the presence of this polymorphism the transport of bile acids mediated by OATP-8 was abolished, but the transport of other substrates wasn't affected, showing that this SNP plays a role in the alteration of the substrate selectivity of the protein (Letschert *et al*, 2004). This may indicate that this region is important for substrate selectivity and alteration in this region may be accompanied by a loss of transport activity of some specific substrates (Letschert *et al*, 2004).

T334G and G699A were described as being in linkage disequilibrium in Japanese patients with renal failure (Tsujimoto *et al*, 2006). Our results suggest a tendency for these SNPs as being in linkage disequilibrium for the population of healthy individuals from Portugal but didn't indicate the same correlation for the populations of healthy individuals from Mozambique and Colombia (results not shown).

Paclitaxel is another OATP-8 substrate that has been tested for a pharmacogenetic effect. However, in this case, no association was observed between the clearance of paclitaxel and either the T334G or G699A SNPs (Smith *et al*, 2007), indicating a minor role of these polymorphisms in transport activity of this compound.

An effect of these SNPs on the transport of other compounds cannot, however, be ruled out.

As the allelic frequencies of T334G and G699A were find to be high, among the different populations analysed it's important to continue to study how these SNPs affect transport activity, since this transporter is involved in the uptake of several clinical important drugs and could be a possible candidate as therapeutic target.

As OATP-C and OATP-8, OCT1 is primarily expressed in the basolateral membrane of hepatocytes (Katsura and Inui, 2003). The gene that encodes this uptake transport, *SLC22A1,* is also polymorphic and some SNPs have been characterized *in vitro* for change of function and for ethnic variability (Shu et al, 2007; Shu *et al*, 2003; Sakata *et al*, 2004; Kerb *et al*, 2002; Itoda *et al*, 2004).

In this work we analysed *SLC22A1* C480G (F160L) and the intronic variants C886G and T4215C (Results - Paper I). These intronic variants had a high and similar allelic frequency among the populations analysed (Portugal: C886G – 16.3%, T4215C – 31.5%; Mozambique:

C886G – 17.5%, T4215C – 20.2%; Colombia: C886G – 17%, T4215C – 30.2%) and to our knowledge this is the first description of these intronic polymorphisms for *SLC22A1.* Although the functional relevance of these intronic polymorphisms is yet to be demonstrated, they may assume a high importance in gene expression regulation, as they could affect the mRNA splicing, give origin to premature STOP codons and give origin to non coding transcripts.

The allelic frequency obtained for C480G (Phe160Leu) in the Portuguese (22.3%) and Colombian (20.3%) populations was significantly higher than in Mozambicans (1.3%), indicating this SNP as ethnicity dependent. Our results are in agreement with another study in a Caucasian population (22%), but are higher than reported to European Americans (6.5%) in another study (Kerb *et al*, 2002; Shu *et al*, 2003). Our results agree with those of Shu et al in the fact that African Americans showed the smaller prevalence (0.5%), and that Mexican Americans showed a frequency similar to European Americans, (5%). These differences may be due to sample sizes and to the origin of the individuals. The similar frequency between Portuguese and Colombians is consistent with the Iberic ancestry of a wide percentage of the Colombian population. This polymorphism is localized in the middle of the second transmembrane domain (Sakata *et al*, 2004). Although some authors had reported no changes in substrate affinity and selectivity, which could explain the high frequencies observed in Portuguese and Colombians, recently an association was described between this polymorphism and the high rate of loss of response or treatment failure with some therapeutic compounds used in the treatment of myeloid leukemia (Kerb et al, 2002; Sakata et al, 2004; Kim et al, 2009), which may indicate that an important role is played by the region where this polymorphism is present. Besides C480G, the variants C41T, C566T, G1201A and 1256delATG have also been associated with decreased uptake activity, in this case of the substrate metformin and with a decrease of metformin ability to lower glucose levels in healthy volunteers (Kimura *et al*, 2005). Also these variants were found to be associated with an increase in area under curve and peak concentration and a lower apparent volume of metformin distribution (Shu *et al*, 2008).

Further association studies are necessary to establish the role of *SLC22A1* polymorphisms in the uptake of endogenous compounds and xenobiotics. However, the first results that show a role in the uptake of therapeutic substrates, and the ethnic variability seen in C480G, should be taken into account if *SLC22A1* is considered as a therapeutic target.

The gene that encodes NTCP, *SLC10A1* is also polymorphic and polymorphisms have been described in different ethnic groups that were shown to interfere with bile acid transport *in vitro* (Ho *et al*, 2004). However, the bile acid phenotype associated with these polymorphisms was not confirmed *in vivo*. In this work we analysed *SLC10A1* C800T (S267F) and the intronic variants A2537G and A2192G (Results - Paper I). While the polymorphism in the coding region was not found in any of the individuals analysed, the intronic variants had a high allelic frequency among the populations studied (Portugal: A2537G – 70.7%, A2192G – 34.8%; Mozambique: A2537G – 98% (significantly higher than Portugal and Colombia), A2192G – 24.2%; Colombia: A2537G – 76.4%, A2192G – 23%). To our knowledge this is the first

description of these intronic polymorphisms for *SLC10A1*. Contrary to other influx transporters, polymorphisms in the coding region of *SLC10A1* are relatively rare, suggesting evolutionary conservation and a key role in the transport of endogenous compounds, making it fundamental to study the non-coding region.

The absence of *SLC10A1* C800T (Ser267Phe) is in agreement with the reported by Ho *et al*, that didn't find this mutation in European American, African American or Hispanic American populations (Ho *et al*, 2004). This polymorphism is predicted to be localized on the third extra-cellular loop, an area of the protein considered a signature motif of NTCP transporters and was associated with a great reduction in transport activity for taurocholate and cholate, compared with the wild-type allele, but fully normal uptake activity was reported for non-bile acid substrates (Ho *et al*, 2004). This variant was also associated with a marked gain of rosuvastatin transport compared with wild-type alleles (Ho *et al*, 2006). According with Ho *et al*, 2004 *SLC10A1* C800T is ethnic-dependent, since this variant only was observed in Chinese Americans (Ho *et al*, 2004). Besides C800T, another variant T668C (Ile223Thr) may count as the polymorphisms more clinically relevant in *SLC10A1*. Ile223Thr variant involves a change from a hydrophobic amino acid to a polar one, predicted to lie within the putative sixth transmembrane-spanning domain, and leads to a global reduction in transport activity (Ho *et al*, 2004). Our results underline an important role for C800T in bile acid substrate recognition, whose presence result in a great reduction of bile acid transport.

Our results contribute to a better understanding of the genetic profile of three populations from different geographical backgrounds, this work being the first description for some of the SNPs analysed. Besides, the majority of the polymorphisms described here are clinically important. In fact, we find some ethnicity-dependent polymorphisms (*SLCO1B3* T334G; *SLCO1B1* A388G, C463A and T521C; *SLC22A1* C480G; *SLC10A1* A2587G), which may contribute to the variability in the treatment with some drugs, such as statins and should contribute to a personalized therapy according with the genetic profile of the population and/or the patient.

Drug metabolizing Enzymes

Polymorphisms in CYP genes are known to affect the fate of cholesterol and to enhance the atherosclerotic process. However, except for the *CYP7A1* A-203C polymorphism and some cerebrotendinous xanthomatosis associated polymorphisms in *CYP27A1*, there are not much information about *CYP7A1, CYP7B1* and *CYP27A1*. In fact, except for *CYP7A1* C-496T and A-203C, and for *CYP7B1* C-116G, the polymorphisms analysed in this section, to the best of our knowledge, represent the first genetic distribution on the populations studied. Therefore, the results obtained in this work contribute for a better understanding of the pharmacogenetic traits of individuals from different geographical locations.

For *CYP7A1* we analysed A698G (N233S), G1039A (G347S), C-346T, C-496T and A-203C polymorphisms (Results - Paper II). From these, only A698G was not detected in any individual analysed from Portugal, Mozambique or Colombia. This polymorphism is localized in exon 3 and leads to an amino acid change from an Asparagine to a Serine and its absence may indicate that it is a conserved region of the gene and its substitution might lead to a non-functional protein. Polymorphisms in the coding region, which result in amino acid alterations, may influence enzyme activity or substrate specificity according with the localization of the SNP.
While G1039A was a rare polymorphism among the populations analysed (Portugal – 0.5%, Mozambique – 2.3%, Colombia – 0.5%), C-346T was a more frequent polymorphism (Portugal – 29.3%, Mozambique – 13.9% (significantly lower), Colombia – 24.2%). The differences observed in the frequency of these polymorphisms may be due their locations, since G1039A is located in the coding region, in exon 4 and results in an amino acid change, from a glycine to a serine. Glycine is an important amino acid for proper folding of the protein and it is known to be a helix breaker and folding blocker of proteins, so, its substitution may lead to conformational changes of the enzyme, which can result in altered catalytic activity. The importance of these regions to obtain a functional and well folded enzyme can be the reason why the polymorphisms in exons 3 and 4 are relatively rare.
On the other side, C-346T is located in the promoter region of *CYP7A1*, between C-496T and A-203C polymorphisms. These three polymorphisms are located in a region that contains several cell-specific regulatory elements whose activity is controlled by nuclear factors, being conceivable that these polymorphisms might alter the transcriptional activity of CYP7A1.
In fact, as happens to C-346T, C-496T and A-203C were also frequent polymorphisms in the populations in this study (Portugal: C-496T – 36.9%, A-203C – 41.8%; Mozambique: C-496T – 50%, A-203C – 52.6%; Colombia: C-496T – 31.9%, A-203C – 40.7%). The results obtained for C-496T are in agreement with another study for Caucasians (United States residents with northern and western ancestry) (36.7%) and Nigerian (55%) populations (Nakamoto *et al*, 2006). The A-203C polymorphism has been the most extensively studied, and several publications have reported allelic frequencies between 36.7%-48% in Caucasians (Hofman *et al*, 2004; Hofman *et al*, 2004a; Kajinami *et al*, 2004; Kajinami *et al*, 2004a; Juzyszyn *et al*, 2008; Nakamoto *et al*, 2006). Our results are inside this range. However, the same doesn´t happen when comparing Mozambicans with other African populations (Nigerians – 58.3%; African Americans – 58%) or Colombians with Mexican Americans (24%), in the same study (Nakamoto *et al*, 2006). The differences observed may be due the sample size of the studies, but most probably due the origin of the individuals, since Africa has different ethnic groups according with the geographic localization and Nigeria is located in Guinea Golf in the West Coast, while Mozambique is located in the East Coast. While the populations from both Colombia and Mexico are genetically admixed, in Colombia the contribution of elements of African ancestry is very important, especially in some regions of the Pacific Coast. This African influence together with the Spanish ancestry explains the higher frequency observed for this polymorphism in Colombia.

Genetic variations in *CYP7A1* have been associated with metabolic disorders of cholesterol and bile acids, including hypercholesterolemia, hypertriglyceridemia, atherosclerosis and gallstone disease (Barcelos *et al*, 2009). Polymorphisms that affect phenotype, by reducing CYP7A1 activity could impede oxysterol generation and LXR-mediated activation of cholesterol transporters, such as ABC transporters among others (Luoma, 2008). Besides, humans lacking CYP7A1, as result of a mutation in this gene have significant elevation of total LDL cholesterol concentrations, substantial accumulation of cholesterol in the liver and a markedly decreased rate of bile acid excretion (Pullinger *et al*, 2002).

Diet influences plasma lipid concentrations and the extension of this influence differs significantly between individuals. In a study published by Barcelos *et al*, an association was demonstrated between the A-203C polymorphism and a reduction in triglyceride concentration after a reduced-fat diet (Barcelos *et al*, 2009). Others studies have also found a relationship between triglyceride concentrations and the A-203C polymorphism (Hofman *et al*, 2004; Hubacek *et al*, 2003; Hofman *et al*, 2004a; Kovar *et al*, 2004; Abrahamsson *et al*, 2005).

Hubacek *et al*, described a significant decrease in plasma total cholesterol after a reduced dietary fat intake over an 8-year follow-up period (Hubacek *et al*, 2003). However, A-203C polymorphism was also associated with increased progression of atherosclerosis and a possible risk of cardiovascular events, in men with coronary atherosclerosis (Hubacek *et al*, 2003). In another study, the risk of a new clinical event after 2 years of treatment with pravastatin was higher in patients with the CC genotype in comparison with patients with the AA and AC genotypes, which may indicate that patients with the polymorphism have a lower benefit from a statin therapy (Hofman *et al*, 2005). Another polymorphism candidate to play a significant role in CYP7A1 activity is C-469T, also located in the promoter region of *CYP7A1.* Wang *et al*, described an association between this polymorphism and plasma LDL cholesterol levels and since then several studies have been conducted to verify the importance of both SNPs, A-203C and C-469T, in cholesterol metabolism regulation (Abrahamsson *et al*, 2005; Wang *et al*, 1998; Couture *et al*, 1999). However, the results obtained were controversial; in a study performed by Abrahamsson *et al*, which included two small cohorts and *in vitro* experiments, these authors concluded that these polymorphisms did not contribute to CYP7A1 activity neither to the plasma LDL-cholesterol concentration (Abrahamsson *et al*, 2005). Lenicek *et al*, reported an association of the CYP7A1 activity with the A-203C substitution in patients after resection of distal ileum (Lenícek *et al*, 2008). The hypothesis proposed from this study was that under physiological conditions, the functional reserve of CYP7A1 is sufficient to override the effect of the change in the promoter region. However, in pathological situations, when the activity of CYP7A1 is up-regulated there is an association between the genotype -203AA and a higher activity of CYP7A1 compared with individuals with the genotype -203CC (Lenícek *et al*, 2008). After statin therapy, this polymorphism was associated with LDL-lowering response (Kajinami *et al*, 2004; Kajinami *et al*, 2004a), indicating a potential role in the efficacy of statin treatment.

Our results for polymorphisms located in coding and promoter regions suggest a different frequency according with the mutation localization, since promoter variants were more frequent

than coding variants. While polymorphisms in the promoter region can modify the degree of gene expression, polymorphisms in coding region may have a direct effect in enzymatic activity altering drug pharmacokinetics, which is confirmed by several studies presented before.

Although polymorphisms in *CYP7B1* have been associated with SPG5A (spastic paraplegia autosomal recessive type 5A) and prostate cancer (Jakobsson *et al*, 2004). and the absence of this enzyme is the cause of CBAS3 (congenital bile acid synthesis defect type 3), there is little information about the characterization of *CYP7B1* SNPs (Wilkinson *et al*, 2003; Tsaousidou *et al*, 2008; Setchell *et al*, 1998).

In this work we analyzed *CYP7B1* G971A (R324H), C1774T and C-116G (Results - Paper II). To our knowledge, this is the first description of the frequencies for the *CYP7B1* variants G971A and C1774T. While C1774T was not found in the populations analyzed, G971A was a rare polymorphism in Portugal (3.3%) and Colombia (4.9%), being absent in Mozambique.

C1774T is localized in the 3'-untraslated region (3'-UTR) and its absence indicates a key role in the gene function which may lead to a lack of expression.

G971A is localized in the coding region, in exon 4, and leads to a change from an Arginine to a Histidine (R324H), which can alter the proper folding of the protein, since Arginine can form salt bridges with Aspartic Acid and Glutamic Acid residues to stabilize the tertiary structure of the protein and Histidine doesn't have this property.

C-116G was a rare polymorphism in Portuguese (4.3%) and Colombian (3.3%), but significantly higher in Mozambicans (13.9%), indicating an ethnicity dependence of this variant, which is in agreement with a previous study (Jakobsson *et al*, 2004). In fact, this was the only report we found for this polymorphism, describing a Swedish population with an allelic frequency (4.04%) similar to our results for the Portuguese population (Jakobsson *et al*, 2004). This polymorphism is localized in the promoter region of *CYP7B1* being associated with an altered promoter activity (Jakobsson *et al*, 2004). Inter ethnic differences may raise the question for a role of this SNP in the development of some diseases, since the incidence of some pathologies differs among populations with different geographical backgrounds.

Polymorphisms in *CYP27A1* have been best described as leading to cerebrotendinous xanthomatosis. Over 40 different mutations in this enzyme have been found to cause this disease, which presents a phenotypic heterogeneity among patients (Cali *et al*, 1991a; Verrips *et al*, 2000; Lee *et al*, 2001; Mast *et al*, 2006).

Here we analysed *CYP27A1* C490T (R164W), C506T (A169V), G817A (D273N) and T1633C (V400A) polymorphisms (Results - Paper II). To the best of our knowledge, this is also the first description of these SNPs. Except for G817A, for which our study unveiled differences among populations with variable ethnic backgrounds (Portugal – 6.5%; Mozambique – 14.6%; Colombia – 17%, Portuguese significantly lower than Mozambicans and Colombians), all the variants were found to be rare (Portugal: C490T – 1.1%, C506T – 2.7%, T1633C – 1.6%;

Mozambique: C490T – 5.3%, C506T – 4.6%, T1633C – 4.3%; Colombia: C490T – 2.2%, C506T – 1.1%, T1633C – 2.2%), with no ethnic differences.
C490T and C506T are located in exon 3 and appear to be located in C-helix α, which is involved in conformational structure of the protein, indicating a potential role in folding and stabilization of the conformational structure of CYP27A1, for these polymorphisms (Sawada *et al*, 2001).
G817A and T1633C are located in exon 4 and appear to be involved in the third and fifth substrate recognition sites, respectively (Sawada *et al*, 2001). The low frequencies of these polymorphisms may suggest that these regions are important for the correct function of the enzyme, with an important degree of conservation.

The majority of the polymorphisms described in this work for drug metabolizing enzymes were analyzed for the first time and distinct distributions could be identified according with the geographic background. Since CYPs play key roles in the metabolism of several endogenous and exogenous compounds and as they are under tight regulation, it's essential to know how genetic variants affect the enzyme capacity of metabolism. In this way, further studies are necessary in this field to establish correct associations between genetic variants and metabolic activity.

Efflux transporters

Mutations in *ABCB11* are the cause of diverse inherited forms of liver disease, including Progressive Familial Intrahepatic Cholestasis Type 2 (PFIC2) or Benign Recurrent Intrahepatic Cholestasis Type 2 (BRIC2) (Jansen and Sturm, 2003). The polymorphisms associated with PFIC2 seem to impair BSEP synthesis, cellular trafficking or stability, while BRIC2 is associated with milder forms of liver disease and the polymorphisms associated with this disease are missense mutations (Strautnieks *et al*, 2008). However, some missense mutations cause severe forms of BSEP deficiency by altering pre-mRNA splicing (Byrne *et al*, 2009). Polymorphisms associated with milder disease may also confer increased risk for development of acquired forms of cholestasis, including intrahepatic cholestasis of pregnancy (ICP) and drug-induced cholestasis (Pauli-Magnus and Meier, 2006; Dixon *et al*, 2009). A number of different mutations in *ABCB11* have been found and functionally characterized *in vitro*. Some of these polymorphisms were found to lead to reduced transport activity, as a result of decreased protein expression, altered membrane targeting or increased degradation by the proteasome (Wang *et al*, 2002b; Hayashi *et al*, 2005; Kagawa *et al*, 2008), while other variants were functional but unstable due to defective glycosylation (Plass *et al*, 2004).
In this study, we determined the distribution of *ABCB11* T580C (S194P), G677A (G260D), C1331T (V444A) and A2155G (M677V) (Results - Paper III). From these, G677A was absent in all individuals analysed and T580C was absent in the Portuguese population and it was rare among Mozambicans (2%) and Colombians (1.1%). Our results for the Portuguese population are in agreement with another study on Caucasians, the only description found for this

polymorphism (Pauli-Magnus *et al*, 2004). These two polymorphisms are located in exons 7 and 8, respectively and they have been associated with an acquired cholestasis, BRIC2 (Stieger *et al*, 2007).

C131T (Portugal – 58.2%; Mozambique – 61.6%; Colombia – 52.7%) was found to be a frequent mutation, while A2155G displayed smaller prevalence (Portugal – 4.3%; Mozambique – 13.2% (significantly higher); Colombia – 6.6%). Our results are in agreement with other studies, where the distribution among Caucasians ranges from 57 to 59.5% for C1331T and from 4 to 4.2% for A2155G, and for an African American population the results obtained were 65.6% and 14% for C1331T and A2155G, respectively (Pauli-Magnus *et al*, 2004a; Meier *et al*, 2006; Lang *et al*, 2006). C1331T is localized in exon 13 and A2155G is localized in exon 17, both originating amino acid changes, V444A and M677V, respectively. C1331T was found in a patient with progressive intrahepatic cholestasis (Hsu *et al*, 2009). Although valine and alanine have similar chemical properties, the valine at position 444 is highly conserved in different mammalian species, indicating that a valine at this position is important for the correct BSEP function (Meier *et al*, 2006). Indeed, *ABCB11* haplotypes containing two alanines are more frequent in patients with primary cirrhosis or associated with higher Mayo Risk Scores in patients with primary sclerosing cholangites (Pauli-Magnus *et al*, 2004a).

The polymorphisms T580C (S194P), C1331T (A444V) and A2155G (M677V) are located in intracellular loop of BSEP and S194P seems to be primary sclerosing cholangitis specific, while the genotype 444A of *ABCB11* was associated with low or very low expression levels of BSEP, which can be a risk factor for the development of acquired forms of cholestasis, such as by drugs or pregnancy (Stieger *et al*, 2007; Pauli-Magnus *et al*, 2004a). Alignment of mammalian *ABCB11* genes shows that the S194P, G260D and V444A variants are in codons for an evolutionary conserved amino acid (Pauli-Magnus *et al*, 2004a).

The association between these genetic variants and some clinical events, together with the differences we find in their distribution suggest that some populations, in this case Mozambicans that had the higher frequencies, are in higher risk of develop some pathologies associated with cholesterol metabolism.

Mutations in *ABCG5* and *ABCG8* genes cause β-sisterolemia, which is characterized by the accumulation of plant sterols in blood and other tissues due the enhanced absorption from the intestine and decreased removal in bile (Berge *et al*, 2000). Several polymorphisms and haplotype structure have been reported together with gene mapping studies and association studies for particular SNPs (Hazard and Patel, 2007). It is hypothesized that these polymorphisms are associated with increased cholesterol excretion into bile resulting in cholesterol supersaturation and gall-stone formation (Wittenburg *et al*, 2005; Buch *et al*, 2007; Gruhage *et al*, 2007; Wang *et al*, 2007).

In the present study we analysed *ABCG5* G80C (G27A), C148T (R50C), C1550G (T517S) and C1810G (Q604E) and *ABCG8* G55C (D19H), A161G (C54Y), C1199A (T400K) and T1895C (V632A) (Results - Paper III).

The frequencies determined for *ABCG5* G80C (Portugal – 18%; Mozambique – 9.3%; Colombia – 8.3%, Portuguese significantly higher than Mozambicans and Colombians), C148T (Portugal – 9.8%; Mozambique – 8.3%; Colombia – 6%) and C1550G (Portugal – 9.8%; Mozambique – 8%; Colombia – 12.7 %) constitute the first description in the literature, to our best knowledge. From these, G80C was ethnicity dependent with different distribution between Portugal and Mozambique / Colombia.

ABCG5 C1810G (Portugal – 16.8%; Mozambique – 28.5%; Colombia – 31.3%, Portuguese significantly lower than Mozambicans and Colombians) was a frequent polymorphism, but also affected by the ethnic background of the individuals, our results being in agreement with other published for Caucasians (11-17%), African Americans (33.1%) and Hispanic (32.5%) (Iwona *et al*, 2008; Hubacek *et al*, 2004; Thompson *et al*, 2005; Plat *et al*, 2005). Although some studies have tried to establish an association between this polymorphism and cholesterol metabolism, the results obtained were contradictory (Weggemans *et al*, 2002; Berge *et al*, 2002; Gylling *et al*, 2004; Kajinami *et al*, 2004).

Although in this work we find statistically significant differences among populations, indicating that G80C and C1810G are dependent on ethnicity, further association studies need to be performed to establish the association between these variants and ABCG5 function.

In the case of *ABCG8*, we observed significantly lower frequencies in the Mozambican population for G55C (Portugal – 7.6%; Mozambique – 1.9% (statistically significant); Colombia – 5.5%), A161G (Portugal – 38.5%; Mozambique – 18.9% (statistically significant); Colombia – 31.9%) and T1895C (Portugal – 23.9%; Mozambique – 8.2% (statistically significant); Colombia – 18.7%) an indicative that, again, ethnicity is important, while C1199A (Portugal – 18.5%; Mozambique – 21.9%; Colombia – 28.6%) revealed a similar distribution among the different populations. Our results are in agreement with other published distributions for Caucasians (G55C: 5.4-9%; A161G: 37.4-43.2%; T1895C: 21-25.3%; C1199A: 15-25%), African Americans (G55C: 1.4%; A161G: 23.1%; T1895C: 5.9%; C1199A: 24%) and Hispanics (G55C: 6.6%; A161G: 32.5%; T1895C: 18.7%; C1199A: 30.1%) obtained in different studies (Iwona *et al*, 2008; Hubacek *et al*, 2004; Koeijvoets *et al*, 2008; Thompson *et al*, 2005; Plat *et al*, 2005).

According with Hubacek *et al*, *ABCG8* C1199A and A161G genotypes influence plasma total cholesterol in a gender-specific manner following dietary intervention (Hubacek *et al*, 2004). In addition, these polymorphisms may increase the risk of gallstone disease (Gruhage *et al*, 2007). The polymorphisms G55C (D19H), A161G (C54Y), C1199A (T400K) and T1895C (V632A) have been associated with several factors affecting cholesterol metabolism, such as baseline cholesterol levels, cholesterol absorption or responsiveness to dietary intervention, indicating a crucial role for these two transporters in cholesterol homeostasis (Iwona *et al*, 2008). Carriers of D19H mutant allele present an up-regulated synthesis of cholesterol (Berge *et al*, 2002; Gylling *et al*, 2004). This can result in a higher efficiency of statin therapy, which is in agreement with the results obtained by Kajinami *et al*, which associate the mutated allele of D19H to a greater plasma LDL-cholesterol reduction after atorvastatin treatment, in comparison with the wild-type

allele (Kajinami *et al*, 2004). Also, the mutant allele of D19H and T400K might help to predict gallstone disease risk, as the mutant allele of D19H was associated with gallstone disease, suggesting that this variant may confer a more efficient transport of cholesterol into bile (Buch *et al*, 2007) and male carriers of the mutant allele of T400K were more susceptible for gallstone disease, compared with wild-type carriers, with no association found in female carriers (Wang *et al*, 2007).

According with the previous reports and our results which indicate a dependent ethnicity distribution for G55C (D19H), statin therapy should be more efficacious in Portuguese and Colombian populations than in Mozambicans. Besides, A161G (C54Y) and T1895C (V632A) had also a different distribution for Mozambique compared with Portugal / Colombia, both with a similar distribution for all these analysed SNPs.

As happened for influx transporters and drug metabolizing enzymes, our results suggest different SNP distribution according with the geographical background of the population. It was the first description of some of the ABC variants analysed here, further studies being necessary to clearly establish if a correlation exists with the clinical outcome, since these genes are potential important targets for the disease characterization or in the development of therapeutic drugs.

The results obtained in this study revealed significant differences in the distribution of polymorphic variants in Cytochrome P450 and membrane transporter genes, which strongly supports the importance of the pharmacogenetic characterization of different populations or ethnic groups. The information gathered in this and other studies should provide genetic markers for a future association with altered clinical parameters with relevance in cardiovascular diseases.

2. Expression profile of genes involved in drug metabolism and transport in response to cholesterol

The expression of several genes involved in pathological situations may be altered during disease and after treatment with drugs. This may indicate their potential role in the development of several diseases as well as in the treatment of the associated pathologies. The increasing knowledge about the genes associated with the susceptibility, development and progression of a certain disease is fundamental for the correct treatment planning. Besides, the genes related with pathological conditions may also be themselves involved in the response to treatment, since they can be responsible for the transport or metabolism of therapeutic drugs.

The main function of bile acids seems to be to act as metabolic regulatory molecules during the daily feeding-fasting cycle since the flux of bile acids returning from the intestine to the liver is increased after a meal (Hylemon *et al*, 2009). Therefore, the ratio of conjugated bile acids and cholesterol is tightly controlled in the bile, since an excess of biliary cholesterol secretion can increase the risk for cholesterol gallstone formation (Hylemon *et al*, 2009). In the liver, bile acids induce several genes via several nuclear receptors and cell signalling pathways (Hylemon *et al*, 2009). The genes induced by bile acids encode transporters and drug metabolizing enzymes, which are involved in regulating bile acid synthesis, transport, conjugation, lipoprotein synthesis, uptake and metabolism (Lefebvre *et al*, 2009). While the role for bile acids in the regulation of metabolism and transport is well characterized at the molecular level, the behaviour of these pathways in response to cholesterol is still obscure. If metabolism and transport genes are hypothetically regulated by cholesterol, it is conceivable that this may impact not only the homeostasis of endogenous compounds but also the concentrations of therapeutic drugs used in the treatment of cardiovascular pathologies.

Statins are the lipid-lowering drugs mostly used in cardiovascular disease. Besides statins, other drugs used in the treatment of lipid disorders include fibrates, cholestyramine and niacin. These drugs and other compounds activate the transcription of genes involved in lipid metabolism. Statins inhibit HMG-CoA reductase and consequently the cholesterol synthesis and also enhance cholesterol elimination, raise plasma apoA1 and HDL-C and reduce apoB and LDL-C (Luoma, 2008). Statins have been identified as P450 inducing agents and they are ligands of PXR, which is a major mediator of induction of P450 enzymes (Lehmann *et al*, 1998). Lipid-lowering drugs have been shown to activate PPARs and LXR (Fan *et al*, 2004).

In this section we intended to describe the changes that take place in gene expression in human cells after exposure to cholesterol. With that aim, we performed expression profiling studies in cell lines from tissues which play a key role in cholesterol homeostasis, such as liver cells (HepG2 cells) and intestine cells (Caco-2 cells). Prior to the actual cholesterol-exposure experiments, these cell lines were sequentially adapted to a serum-free medium to minimize variables and optimize results, since some compounds, such as cholesterol, which are present in serum, could interfere with the results. The cells were then treated with cholesterol at different concentrations, during different time periods and mRNA was extracted. Expression levels for the selected genes, potentially related to cardiovascular diseases, were evaluated for the definition of response patterns to cholesterol. The selected genes analyzed in this work include metabolizing enzymes (*CYP3A4, CYP7A1, CYP27A1, CYP39A1, CYP51A1, CYP7B1, CYP8B1*), or influx (*SLC10A1, SLC22A1, SLC22A7, SLCO1A2, SLCO1B1, SLCO1B3, SLCO2B1*) and efflux (*ABCA1, ABCB11, ABCC2, ABCC3, ABCC6, ABCG2, ABCG5, ABCG8*) transporters.

Influx transporters

The relevance of solute carriers has been, in recent years, progressively demonstrated with a particular focus on the role of some of these membrane proteins in the transport of several important clinical compounds.

We investigated the response to cholesterol of some uptake transporters, in liver and intestine cell line models. Cholesterol treatment led to an increase in expression of influx transporters *SLC10A1* (NTCP), *SLCO1B3* (OATP-8), *SLCO2B1* (OATP-B) and *SLC22A1* (OCT1) in HepG2 cells (Table 1).

In Caco-2, a less significant effect of cholesterol was observed, since fewer genes were shown to be induced. Interestingly, the concentration of fetal bovine serum present in the culture medium was shown to affect the changes in expression, since only *SLCO1A2* (OATP-A) was induced at 1% serum, while at a higher concentration (20%) changes were seen in the expression of *SLCO1B3* (OATP-8) and *SLC22A7* (OAT2) (Table 1).

Table 1- Effect of cholesterol on the expression of influx drug transporters in HepG2 and Caco-2 cells: The symbol ↑ represents a significant increase of the mRNA levels compared with the time 0h of treatment, while the symbol ↓ represents a significant reduction of the mRNA levels compared with the time 0h of treatment.

Gene name (Protein name)	HepG2 cells (0.05% serum)					Caco-2 cells (1% serum)					Caco-2 cells (20% serum)				
	2h	4h	8h	12h	24h	2h	4h	8h	12h	24h	2h	4h	8h	12h	24h
SLC10A1 (NTCP)			↑												
SLC22A1 (OCT-1)			↑												
SLC22A7 (OAT2)													↓	↓	↓
SLCO1A2 (OATP-A)						↑	↑			↑					
SLCO1B1 (OATP-C)															
SLCO1B3 (OATP-8)	↑		↑		↑										↑
SLCO2B1 (OATP-B)		↑													

In this work, *SLC10A1* expression was increased in response to cholesterol after 8h of treatment, in HepG2 cells, but no significant changes were observed in Caco-2 cells (Results - Paper IV and Paper V). This increase in a liver cell model indicates a positive regulation of this gene by cholesterol or cholesterol-derived compounds. The fact that a similar effect is not

observed in Caco-2 cells suggests that the signalling pathways involved in the activation of this gene by cholesterol may depend on an interaction with a tissue-specific factor.
NTCP, a sodium-dependent transporter, is the main transporter for bile acids, although these compounds can also be transported by OATPs, sodium-independent transporters. However, OATPs seem to have a secondary role in the transport of bile acids (Tirona and Kim, 2002).
Besides being considered the main transporter of bile acids, NTCP is also responsible for the transport of several drugs, including cholesterol-lowering drugs, such as statins, contributing to overall drug uptake into hepatocytes.

SLC10A1 gene expression may be suppressed as an adaptive response to reduce the uptake of bile acids into the hepatocyte. Bile acids feed back to down-regulate the transcription of *SLC10A1* by FXR-dependent and independent mechanisms to avoid cytotoxic bile acids accumulation and for this reason, the activation of FXR serves as a critical modulator of the enterohepatic circulation and *de novo* synthesis of the bile acids to provide tight regulation of the bile acids pool (Lefebvre *et al*, 2009; Dawson *et al*, 2009). Although FXR does not interact directly with the *SLC10A1* promoter, it induces the expression of other factors, such as SHP, RARα:RXRα, HNF-1α, HNF-4α, Foxa2 (HNF-3β) and c-Jun N-terminal kinase (JNK) to indirectly repress *SLC10A1* expression (Karpen *et al*, 1996; Eloranta *et al*, 2006). Therefore, FXR is considered to have a function to ameliorate the liver toxicity of bile acid congestion. In fact, in pathophysiological situations, such as in different forms of cholestatic liver disease, *SLC10A1* transcription is decreased as part of a coordinated response to reduce the extent of liver injury (Geier *et al*, 2007).

Although these reports have described a repression of *SLC10A1 in vivo*, in our cell model, HepG2, we observe an induction of this gene's expression, which may occur by a direct effect of cholesterol or oxysterols in our experimental conditions. One explanation for this contradictory effect is that in *in vivo* models the majority of cholesterol is metabolized to bile acids that can repress some target genes, such as *SLC10A1*, when they are at high levels in the cell, while in HepG2 cells, cholesterol might be less extensively metabolized to bile acids and therefore our results might reflect an effect of cholesterol by itself.

In HepG2 cells, the levels of *SLCO1B3* mRNA increased after 2h of cholesterol treatment and then decreased in the remaining periods of treatment, while in Caco-2 cells this induction was only observed after 24h of cholesterol treatment (Results - Paper V).
SLCO1B1 wasn't responsive to cholesterol in both cell lines, maintaining its expression approximately constant along the period of treatment (Results - Paper V).
OATP-C (*SLCO1B1*) and OATP-8 (*SLCO1B3*) seem to have as binding sites a region rather than a discrete single site and can accommodate numerous structurally unrelated substrates (Miyagawa *et al*, 2009; Gui and Hagenbuch, 2009). OATP-C plays an important role in the uptake of a wide variety of compounds, including bile salts and therapeutic drugs, such as

statins and anticancer drugs. OATP-8 has high homology and overlapping substrate specificity with OATP-C, but OATP-8 was shown to be expressed in cancer cells indicating an important role in the uptake of endo- end xenobiotics into hepatocytes as a backup system for OATP-C and it may also be involved in the uptake of drugs or hormones into cancer cells (Hagenbuch and Meier, 2004).

FXR controls the expression of the OATP family, which suggests a decrease would be observed with biliary congestion (Simon *et al*, 1996). However, OATP-8 in bile acid congestion increases its expression by a direct effect of FXR (Jung *et al*, 2001b). Our observations are in agreement with these results, since we also observed an increase in *SLCO1B3* expression in response to cholesterol. Two hypothetical scenarios may be envisaged: the induction may be mediated by cholesterol itself or an indirect effect via bile acid activated FXR. Although the results obtained for *SLC10A1* pointed to a direct effect of cholesterol in gene expression, the metabolism of cholesterol still happens, although to a less extension. This means that bile acids are being produced, and their concentration in the cell may be enough to activate FXR and consequently *SLCO1B3*.

The fact that *SLCO1B3* induction is an early event in HepG2 cells (2h) while an effect can only be observed after 24h in Caco-2 cells further suggests that we may be in the presence of two distinct mechanisms: the early response in HepG2 suggests a direct effect of cholesterol, while metabolism to bile acids may be needed in Caco-2 cells for FXR activation. Consistent with this hypothesis is the fact that the expression of LXR (especially the alpha isoform) is much higher in the liver, being able to mediate oxysterol effects.

As these uptake transporters seem to have high overlap in terms of substrate specificity, the differences observed in their expression may occur because these transporters can act as a complement of each other. Since *SLCO1B3* expression is normally found in cancer cells, from which our cells derive, induction might be facilitated by interaction of a cholesterol-activated transcription factor with a pre-existing factor in these cells. *SLCO1B3* might be first induced in response to an increase in cholesterol to cope with this challenge. One could think of *SLCO1B1* as a backup system to help *SLCO1B3* when cells are exposed to a high concentration of a substrate (eg. a drug) for which OATP-8 mediated transport may not be sufficient.

For *SLCO1A2*, we observed no significant changes in HepG2 cells in response to cholesterol but in Caco-2 cells (1%FBS) the mRNA levels were induced after cholesterol treatment (Results - Paper IV and Paper V).

As OATP-A (*SLCO1A2*) is ubiquitously expressed it can transport a wide range of endogenous and exogenous compounds, mainly amphipathic, organic compounds. OATP-A is, also, probably involved in renal reabsorption of endo- and xenobiotics (Tirona and Kim, 2002). OATP-A seems to be under PXR control, since, in rat liver, mRNA levels of *SLCO1A2* were reduced in ethinylestradiol-induced cholestasis associated with decreased binding of PXR (Geier *et al*, 2003). These results are in agreement with other from PXR null mice and together they suggest

a coordinate role of PXR in the regulation of *SLCO1A2* and *CYP7A1* (Staudinger *et al*, 2001). Also, HNF-1α is involved in the regulation of *SLCO1A2* (Jung *et al*, 2001a).
From our results, it seems possible that OATP-A has a minor role in the uptake of some compounds, when other transporters are present. We didn't observe a repression, as observed in cholestatic rat liver. One scenario is that cholesterol or one derivative may activate PXR. Another hypothesis is that, since PXR levels are much lower in HepG2 cells than in liver, any decrease in PXR binding would not be detectable.

In the present study, *SLCO2B1* was found to be responsive to cholesterol after 4h of treatment, in HepG2 cells but no changes were observed in Caco-2 cells (Results - Paper V).
Although OATP-B is ubiquitously expressed it has high substrate specificity. Its constitutive expression in liver and intestine is dependent on the transcription factor Sp1, and it is repressed by CAR and AhR in human hepatocytes (Jigorel *et al*, 2006). This is consistent with the results by Stedman *et al*, that PXR and CAR play a key role in the regulation of lipid and bile acids homeostasis (Stedman *et al*, 2005).
The induction of *SLCO2B1* after cholesterol treatment suggests that this uptake transporter is involved in the cholesterol homeostasis in liver. The underlying mechanism probably involves a tissue-specific transcription factor acting in cooperation with a cholesterol-activated regulator.

SLC22A1 gene expression was increased after 8h of cholesterol treatment, in HepG2 (Results - Paper IV). No significant changes were observed in Caco-2 cells, after cholesterol treatment (Results - Paper V).
OCT-1 is an organic cation transporter, expressed in liver and intestine, that mediates the uptake of several endogenous compounds and therapeutical drugs from circulating blood (Katsura and Inui, 2003; Mizuno *et al,* 2003). Several amino acid residues have been determined to be involved in substrate binding, indicating the existence of a substrate-binding region instead of a single binding site for OCT1, and allowing the binding of more than one substrate in this region at the same time (Koepsell *et al*, 2007). Other studies have demonstrated that substrates can access several amino acids from the extracellular or intracellular sides of the plasma membrane and are therefore likely to be part of the translocation pathway (Volk *et al*, 2009).
The *SLC22A1* promoter has two DNA response elements for the liver-enriched transcription factor HNF-4α, involved in the transcriptional activation (Saborowski *et al*, 2006). However, bile acids repress *SLC22A1* transcription through the action of FXR. Bile acid-activated FXR binds SHP, which in turn inhibit the binding of HNF-4α (Saborowski *et al*, 2006). During cholestasis the hepatic expression of OCT-1 is decreased and after bile duct ligation in rat, a model for obstructive cholestasis, OCT-1 is also decreased in liver, but not in kidney, where an up-regulation of this uptake transporter was observed (Denk *et al*, 2004). Cholestasis also can be induced by intraperitoneal application of lipopolysaccharides and, in fact, OCT-1 expression was down-regulated in rats after treatment with this compound (Cherrington *et al*, 2004). In

humans, the increased levels of bile acids after obstructive cholestasis may inhibit HNF-4α mediated activation of *SLC22A1* (Koepsell *et al*, 2007).
However, OCT1 is also positively regulated by some compounds, PPAR-α and –γ agonists, such as clorofibrate and ciglitazone, usually used in the treatment of dyslipidemias and diabetes mellitus (Nie *et al*, 2005). Besides, in humans, the levels of *SLC22A1* and *SLCO2B1* mRNA are also increased in hypercholesterolemic patients compared with normolipidemic individuals and after atorvastatin treatment gene expression was down-regulated (Rodrigues *et al*, 2009). In fact, Rodrigues et al, observed a reduction of LDL cholesterol and total cholesterol serum concentration after atorvastatin therapy (Rodrigues *et al*, 2009).
The induction observed for *SLCO2B1* and *SLC22A1* in HepG2 after cholesterol treatment is therefore in agreement with the increased expression observed *in vivo* in patients with hypercholesterolemia, suggesting an important role for these transporters in cholesterol homeostasis and perhaps as potential targets for therapeutic drugs.

In Caco-2 cells, mRNA levels of *SLC22A7* were increased after 2h of cholesterol treatment and then significantly decreased after 8h, 12h and 24h of cholesterol treatment. No significant changes were observed in HepG2 cells (Results - Paper V).
OAT2 is an organic anion transporter that plays important roles in absorption, distribution and elimination of numerous endogenous and exogenous compounds.
As for the *SLC22A1* gene, *SLC22A7* is also activated by HNF-4α (Popowski *et al*, 2005). *SLC22A7* was down-regulated by bile acids, in Huh7 cells (Popowski *et al*, 2005) and in a rat model of obstructive cholestasis (Denk *et al*, 2004).
Again, induction may be mediated by cholesterol itself or, most probably, an indirect effect via bile acid activated FXR, after conversion of cholesterol to bile acids in Caco-2 cells. In this case, since *SLC22A7* was induced in these cells, one could speculate that a direct positive regulation by FXR may be taking place, independently of the SHP-HNF-4 signaling axis, which normally leads to transcriptional repression.

Our results suggest an important role played by *SLC10A1, SLC22A1, SLBO1B3* and *SLCO2B1* in liver, in response to cholesterol treatment, suggesting a regulatory function of these genes in cholesterol homeostasis. On the other hand, in the intestine, our results suggest a minor role of the previous transporters, except for *SLCO1B3*, which was also responsive in Caco-2 cells, and a potential function of *SLC22A7* and *SLCO1A2*, since these were the most responsive uptake transporters in Caco-2 cells.
Taken together, our results demonstrate that the expression of influx transporters is susceptible to changes when cholesterol concentrations are modified, in liver and intestine-derived cells. Since these proteins are involved not only in the transport of endogenous compounds but also of several therapeutic drugs, our results raise the question of a possible differential response to therapeutics in subjects with pathologies involving changes in cholesterol homeostasis.

Further studies are necessary to better characterize the regulatory mechanisms underlying the observed changes and to clarify if those are due to a direct regulation by cholesterol (e.g. through the nuclear receptor ROR) or indirect via bile acid (FXR/PXR) or oxysterol (LXR) signaling.

Drug metabolizing enzymes

There are about 60 distinct CYP genes in humans (Siest *et al*, 2005). The importance of the different families in drug metabolism has been well documented and the interest for their potential role in therapeutics has been increasing.

In the present work we have shown that the drug metabolizing enzymes which are more responsive to cholesterol, in HepG2 cells were *CYP7A1, CYP27A1* and *CYP7B1* (Table 2).
In Caco-2 cells, the set of genes induced by cholesterol were again dependent on the serum concentration, namely *CYP7A1, CYP7B1, CYP39A1* and *CYP51A1* in the presence of 1% FBS and *CYP3A4, CYP7A1, CYP7B1, CYP27A1* and *CYP8B1* at 20% FBS (Table 2).

Table 2- Effect of cholesterol on the expression of drug metabolizing enzymes in HepG2 and Caco-2 cells: The symbol ↑ represents a significant increase of the mRNA levels compared with the time 0h of treatment, while the symbol ↓ represents a significant reduction of the mRNA levels compared with the time 0h of treatment.

Gene name	HepG2 cells (0.05% serum)					Caco-2 cells (1% serum)					Caco-2 cells (20% serum)				
	2h	4h	8h	12h	24h	2h	4h	8h	12h	24h	2h	4h	8h	12h	24h
CYP3A4 EC1.14.13.97										↓	↓	↓	↓		
CYP7A1 EC1.14.13.17		↑	↑		↓				↑						↑
CYP7B1 EC1.14.13.100		↑	↑			↓	↓	↓	↓	↓				↑	↑
CYP8B1 EC1.14.13.95															↑
CYP27A1 EC1.14.13.15			↑												↑
CYP39A1 EC1.14.13.99						↑	↑	↓	↑	↑					
CYP51A1 EC1.14.13.70						↑				↑					

In the present study, we report a two-step response of *CYP7A1* mRNA to cholesterol in HepG2 cells, being induced at short term treatment and then reduced after longer exposure periods. In

Caco-2 cells an increase in expression of *CYP7A1* was observed after 12h (1%FBS) and 24h (20%FBS) of treatment (Results - Paper V).

CYP7A1 is the first and rate-limiting enzyme in the classical pathway of bile acid biosynthesis (Insull, 2006). This is the main pathway of cholesterol conversion to bile acids; therefore the activity of CYP7A1 is subject to complex modes of control. Bile acids can also be synthesized by an alternative or acidic pathway, which is governed by CYP27A1 and converts oxysterols to bile acids (Insull, 2006; Lefebvre et al, 2009). This is a minor pathway and in normal conditions only 6% of bile acids are synthesized by this pathway. However, in certain conditions, such as fetal development or chronic liver disease this pathway may have a higher contribution to bile acids synthesis (Crosignani *et al*, 2007; Back and Walter, 1980). However, unlike *CYP7A1*, *CYP27A1* is not known to be regulated by bile acids (Lefebvre et al, 2009). *CYP7A1* is repressed in response to the daily-feeding-fasting cycle, resulting in a decrease of the synthesis of bile acids from intrahepatic cholesterol.

Due the importance of CYP7A1 in bile acids synthesis, this gene is highly regulated at the transcriptional level. Besides, the mRNA has a short half-time and possesses an unusually long 3'-untranslated region (3'UTR) (Hylemon *et al*, 2009). *CYP7A1* is repressed by bile acids, glucagon, certain cytokines (TNF-α, IL-1) and FGF 15/19 (Gilardi *et al*, 2007) and induced by thyroid hormone and glucocorticoids (Gilardi *et al*, 2007).

Cholesterol and oxysterols have a controversial role in *CYP7A1* regulation since a wide species-related variability has been reported, a positive effect was observed for cholesterol in rats (Horton *et al*, 1995) and human (Duane, 1993) while a repression was observed in monkeys (Rudel *et al*, 1994) and rabbits (Xu *et al*, 1997).

Our results indicate a two-step response of *CYP7A1* to cholesterol, in HepG2. This may occur because in an initial phase the concentration of cholesterol remains high in the cell and has a positive effect in the regulation of *CYP7A1*. However at longer periods, cholesterol concentration is lower due to its conversion to bile acids, which act as repressors of *CYP7A1*. However, in Caco-2 cells the effect observed is the opposite. One possible explanation is that in the intestine the conversion of cholesterol to bile acids via the classical pathway occurs to a lesser extent (consistent with the fact that intestinal CYP7A1 expression is low), and the induction observed comes from the effect of cholesterol in the cell or, alternatively, from the effect of FXR after bile acid synthesis via the less efficient alternative pathway.

Besides, in a study by Englund *et al*, where the pattern of gene expression was compared between Caco-2 cells and human intestine, the results have shown that Caco-2 expression pattern was clearly distinguishable from that found in human small intestine (Englund *et al*, 2006) but was in good agreement with human jejunum (Hilgendorf *et al*, 2007), which also should help to explain some differences observed in *in vivo* models and in *in vitro* cell lines.

In HepG2 cells, *CYP7B1* was highly responsive to treatment with cholesterol. The levels of *CYP7B1* increased after 4h of treatment and then suffered a reduction. In Caco-2 cells cultured

in the presence of 1% FBS, *CYP7B1* levels were repressed while in the presence of 20% FBS *CYP7B1* mRNA expression was increased (Results - Paper V).

CYP7B1 is another enzyme involved in bile acid synthesis. During bile acid biosynthesis other products can be formed, such as 27-hydroxycholesterol, which is a regulatory oxysterol together with 25-hydroxycholesterol (Luoma, 2008; Fiévet and Staels, 2009). *CYP7B1* is also under control of SHP, which may play a role in down regulation of lipogenesis and protection against steatosis (Boulias *et al*, 2005). In rats and mice *CYP7B1* mRNA, protein levels and enzyme activity were not affected by cholesterol feeding, but were increased in primary rat hepatocytes treated with cholesterol or mevalonate, which suggests that cholesterol may also regulate *CYP7B1* (Ren *et al*, 2003). A report case about a patient lacking CYP7B1 with severe cholestasis, cirrhosis and liver failure, which are symptoms of hepatotoxic effects of high concentrations of side chain hydroxycholesterols and unsaturated monohydroxy bile acids, indicates that the main function of this enzyme is the inactivation of oxysterols, thereby protecting the liver from cholestasis (Setchell *et al*, 1998; Russel, 2003; Norlin and Chiang, 2004).

Our results are in according with these authors that suggest a role for cholesterol in *CYP7B1* regulation, since this gene was highly responsive to cholesterol treatments in this work. As happened to *CYP7A1, CYP7B1* had a two-step response to cholesterol treatment, in HepG2 cells, which may occur by the same reasons, since it was observed an induction followed by an inhibition at the same treatment periods, in both genes. In Caco-2 cells, we observe a different response according with the serum concentration, being *CYP7B1* induced at higher serum levels and repressed at lower serum levels. The induction observed may be a consequence of cholesterol itself together with other compounds present in the serum that act as activators of the *CYP7B1* gene expression, since the exact constitution of the serum is unknown, while the repression observed at low serum levels may be the consequence of cholesterol conversion in bile acids, since the interference of the serum compounds is minimal in this case.

We did not observe any changes in *CYP8B1* mRNA expression in response to cholesterol in HepG2 cells, while in Caco-2 cells (20%FBS) an increase in *CYP8B1* levels was observed after 24h of cholesterol treatment (Results - Paper V).

CYP8B1 is responsible for the conversion of bile acid intermediates to cholic or chenodeoxycholic acid and therefore the cholic acid/chenodeoxycholic acid ratio, determining the hydrophobicity and biological properties of the bile acids pool (Lefebvre *et al*, 2009). As in the case for *CYP7A1*, the transcriptional regulation of *CYP8B1* is also highly controlled by many of the same mechanisms, however with some differences, as these enzymes locate in different compartments (Hylemon *et al*, 2009). *CYP8B1* is under regulation of LRH-1 (Lee *et al*, 2008) and FXR (Yang *et al*, 2002). Some studies in rats have showed that cholesterol, thyroid hormones and insulin decrease mRNA levels while starvation has the opposite effect (Vlahcevic *et al*, 2000).

Our results suggest a minor role of *CYP8B1* in cholesterol regulation, in liver. However, in Caco-2 cells with high serum levels, we observed an induction. This may occur by the same reasons given for *CYP7B1*.

We have shown an induction of *CYP27A1* mRNA levels in Caco-2 cells after 24h of treatment, while in HepG2 cells, there is an early increase in *CYP27A1* mRNA levels after 8h of treatment that decreases thereafter, reaching basal levels after 24h (Results - Paper V).
Conversely to CYP7A1 that is only expressed in hepatocytes, CYP27A1 is ubiquitously expressed, raising the hypothesis of a key role for this enzyme in reverse cholesterol transport (Luoma, 2008). This enzyme catalyses the hydroxylation of cholesterol with the formation of 25-hydroxycholesterol, a potentially important regulatory oxysterol (Li *et al*, 2007). This metabolite can be metabolized, in hepatocytes, to 25-hydroxycholesterol-3-sulfate, which can also be an important regulatory metabolite (Ma *et al*, 2008). Both metabolites activate LXR, an important regulator of cholesterol homeostasis (Fiévet and Staels, 2009; Chen *et al*, 2007). *CYP27A1* is also inhibited by FXR and the promoter has also an HNF-4α binding site (Agellon *et al*, 2002). PXR induces *CYP27A1* expression in intestinal cells but not in liver cells, suggesting that PXR may also regulate *ABCA1* efflux of cholesterol from intestine cells to synthesize HDL for reverse transport of cholesterol to the liver (Li *et al*, 2007). PXR is a master regulator of P450 enzymes in the metabolism of xenobiotics and affects the fate of cholesterol (Chiang, 2003; Eloranta and Kullak-Ublich, 2005). As some reports show differences between changes in mRNA level and CYP27A1 activity, this may suggest that this enzyme is post-transcriptionally regulated (Pikuleva, 2006).
The results obtained for this gene were similar than for *CYP7A1* and *CYP7B1*, in HepG2 cells, which may indicate that these genes play key roles in the homeostasis of cholesterol, regulating its metabolism in liver. *CYP27A1* expression in Caco2-cells had the same expression pattern than *CYP7A1, CYP7B1* and *CYP8B1* suggesting a lower conversion of cholesterol to bile acids and a direct effect of cholesterol in the gene regulation.

In the liver, bile acids inhibit *CYP7A1* and *CYP8B1* through FXR which induces SHP to inhibit gene transcription, while in the intestine FXR induces FGF19 to activate liver FGF receptor 4 signaling to inhibit gene transcription.
CYP7A1 and *CYP8B1* expression exhibits a pronounced diurnal rhythm, with two peaks, in humans, around 3pm and 9pm, which is regulated by Rev-erbα, D-site binding protein (DBP) also named c/EBP-beta, DEC2 and E4BP4 that are clock genes (Kawamoto *et al*, 2006; Yamada *et al*, 2000; Noshiro *et al*, 2007; Noshiro *et al*, 2004). In humans, the activity of CYP7A1 during daytime is independent of food intake and opposite from the circadian rhythm of cholesterol synthesis (Galman *et al*, 2005). HNF-4α is essential for the maintenance of diurnal variation of *CYP7A1* expression, since mice lacking this nuclear factor lose up-regulation during the dark cycle (Inoue *et al*, 2006). FXR and HNF-4α bind to *CYP7A1* and *CYP8B1* promoters,

but the HNF-4α binding site overlaps with a binding site for LRH-1 (Chen and Chiang, 2003). Thus, FXR and HNF-4α may coordinately regulate bile acid synthesis and conjugation.
PPARα is also important in the regulation of bile acids synthesis, since bile acids induce PPARα transcription via induction of FXR (Pineda Torra *et al*, 2003). PPARα regulates *CYP7A1* and *CYP27A1* transcription by inhibiting HNF4α transactivation activity, indicating that PPARα may play a role in balancing the amount of conjugated and free bile acids (Marrapodi and Chiang, 2000).
It was described that loss of RORα and/or RORγ had major effects on the expression of drug metabolizing enzymes and transporters (Wada *et al*, 2008). In RORα/RORγ double knockout mice *CYP7B1* and *CYP8B1* have been shown to be suppressed. However, further studies indicated only RORα as a positive regulator of *CYP7B1*, suggesting a selective control by RORα or RORγ (Kang *et al*, 2007). Moreover, RORα null mice showed lower plasma triglyceride and cholesterol levels than control mice, suggesting a regulatory role for RORα in the homeostasis of cholesterol, oxysterols and bile acids (Wada *et al*, 2008).

No changes in *CYP3A4* levels were observed in HepG2 cells after cholesterol treatment (Results - Paper IV). However, *CYP3A4* was responsive to cholesterol treatment in Caco-2 cells. While in Caco-2 cells in the presence of 1%FBS, *CYP3A4* mRNA expression was increased after 24h of treatment, in Caco-2 cells 20%FBS a repression first occurred after 2h of treatment and then an increase was observed for longer periods of cholesterol treatment (Results - Paper V).
CYP3A4 is involved in the conversion of cholesterol to 4β-hydroxycholesterol (Pikuleva, 2006) and is a key enzyme in the metabolism of a broad range of drugs, affecting their pharmacokinetic and pharmacological activities (Ribeiro and Cavaco, 2006). This enzyme metabolizes about half of all pharmaceutical compounds. Although CYP3A4 does not play a major role in cholesterol catabolism it may be important in transcriptional regulation of lipid metabolism. Secondary bile acids are regulated by VDR in liver and intestine, mediating the expression of *CYP3A4* and consequently its detoxification (Luoma, 2008). Bile acids, statins, epoxycholesterol metabolites of cholesterol among other structurally diverse compounds can activate *CYP3A4* and other P450 enzymes via PXR (Lehmann *et al*, 1998; Cali *et al*, 1991a). FXR also induces expression of *CYP3A4*, which in turn detoxifies bile acids by oxidizing them (Luoma, 2008). Oxysterols are endogenous signal compounds, which via LXR induce genes involved in cholesterol efflux, transport, excretion and absorption (Tontonoz and Mangelsdorf, 2003). Free cholesterol and cholesterol esters don't have the ability to activate these genes, so it is necessary that hydroxylation of cholesterol occurs. *CYP7A1, CYP27A1* and *CYP3A4* are the most important isoenzymes involved in the formation and metabolism of oxysterols and for this reason their regulation is highly controlled.
In fact, our results suggest that *CYP3A4* plays a role in cholesterol homeostasis in intestine, since this gene was responsive to cholesterol treatment. The late induction observed is consistent with the known FXR-mediated induction of *CYP3A4*, which would depend on bile

acid production. The very early repression observed in the presence of a high concentration of serum is intriguing, and suggests an interaction with a factor present in the serum. However, at this phase our results do not allow us to draw any further conclusion.

No changes in *CYP39A1* mRNA were observed in HepG2 cells in response to cholesterol, but in Caco-2 cells (1%FBS) a variation in the pattern of expression was observed in response to treatment, starting with an increase till 4h, and then a decrease in *CYP39A1* levels at 8h of treatment followed by another increase at longer periods of cholesterol treatment (Results - Paper V).
In the liver, CYP39A1 is responsible for the conversion of 24S-hydroxycholesterol to bile acids (Björkhem, 2006). *CYP39A1* mRNA is abundantly and constitutively expressed in liver (Li-Hawkins *et al*, 2000). *CYP39A1* mRNA hepatic levels have been shown in mice to remain unchanged in response to dietary cholesterol, bile acids and a bile-acid binding resin, in agreement with our observations (Pikuleva, 2006).
While in HepG2 cells, *CYP39A1* seems to not contribute for the control of cholesterol concentrations, in Caco-2 cells *CYP39A1* gene was responsive to cholesterol. However, as happened with *CYP3A4*, this response was variable according with the period of treatment. Not much is known about the regulation of *CYP39A1*, but we can speculate that these changes in expression are probably due, as discussed for influx receptors, to changing concentrations of the endogenous compounds which are acting as regulators, and to a changing role of nuclear receptors ROR, FXR and PXR in the control of *CYP39A1* expression.

CYP51A1 was not responsive to cholesterol treatment in HepG2 cells. However in Caco-2 cells (1%FBS) *CYP51A1* levels were shown to change, with two peaks of significantly increased expression after 2h and 24h of cholesterol treatment (Results - Paper V).
CYP51A1 is a ubiquitously enzyme and the only CYP participating in cholesterol synthesis (Gibbons, 2002). Oxysterols are subproducts of the reaction of demethylation, catalyzed by this enzyme, which are involved in the inhibition of HMG-CoA reductase and sterol synthesis (Gibbons, 2002; Saucier *et al*, 1989). During cholesterol synthesis is formed 24S, 25-epoxycholesterol, and an activator of LXR that may act against cholesterol accumulation (Lehmann *et al*, 1997). In humans, low levels of cholesterol increases *CYP51A1* mRNA, indicating that the regulation of this enzyme occurs essentially at transcriptional level (Pikuleva, 2006).
The same suggestion may be raised, as in the case of *CYP39A1*, that variable expression levels during exposure to cholesterol may be related to changing concentrations of the endogenous regulators. The role of nuclear receptors activated by cholesterol, oxysterols and/or bile acids in *CYP51A1* as well as in *CYP39A1* regulation remains to be established.

Our results suggest an important role played by *CYP7A1*, *CYP7B1* and *CYP27A1* in liver, in response to cholesterol treatment, suggesting a key regulatory function of these enzymes in

cholesterol metabolism. On the other hand, in intestine, our results suggest the participation of all CYPs studied, to variable extents.

ABC transporters

ATP-binding cassette transporters constitute a large superfamily of active transporters, which in mammals is constituted by seven families coding for 48 individual transporters. ABC transporters have been extensively studied and its role in disease risk, in response to existing drugs as well as in the development of new therapeutics is well established.

Cholesterol treatment led to an increase in expression, of efflux transporters *ABCB11* (BSEP), *ABCC6* (MRP6), *ABCG5* and *ABCG8* in HepG2 cells (Table 3).

In Caco-2 cells in the presence of 1% FBS, cholesterol treatment led to an increase in expression of ABC transporters *ABCA1* and *ABCG8* and in Caco-2 cells at 20% FBS, of *ABCB11* (BSEP), *ABCC2* (MRP2), *ABCC6* (MRP6) and *ABCG5* (Table 3).

Table 3- Effect of cholesterol on the expression of efflux drug transporters in HepG2 and Caco-2 cells: The symbol ↑ represents a significant increase of the mRNA levels compared with the time 0h of treatment, while the symbol ↓ represents a significant reduction of the mRNA levels compared with the time 0h of treatment.

Gene name (Protein name)	HepG2 cells (0.05% serum)					Caco-2 cells (1% serum)					Caco-2 cells (20% serum)				
	2h	4h	8h	12h	24h	2h	4h	8h	12h	24h	2h	4h	8h	12h	24h
ABCA1 (ABC1)									↓						
ABCB11 (BSEP)		↑	↑		↑										↑
ABCC2 (MRP2)				↑											
ABCC3 (MRP3)															
ABCC6 (MRP6)					↓						↓		↓		↑
ABCG2 (BCRP)															
ABCG5 (sterolin)		↑	↑										↑		↑
ABCG8 (sterolin)	↑	↑	↑						↑						

ABCA1 mRNA expression only was altered in Caco-2 cells (1%FBS) with a decrease after 12h of cholesterol treatment, with no significant changes in HepG2 cells (Results - Paper V).

ABCA1 has a key role in the regulation of cellular lipid efflux (Luoma, 2008). In the liver it is the main factor in the generation of HDL-C in circulation, while in intestine it is only a contributory factor (Brunham *et al*, 2006). Intestinal ABCA1 controls the absorption of cholesterol by effluxing it from the enterocytes back to the intestinal lumen (Repa *et al*, 2000). Enterocytes deficient in ABCA1 absorb smaller amounts of cholesterol and thus, HDL contributes to cholesterol absorption from the intestine (Brunham *et al*, 2006). *ABCA1* is regulated by LXR and statins can inhibit the synthesis of oxysterol ligand for LXR and down-regulate *ABCA1* and cholesterol efflux (Ando *et al*, 2004). Statin-mediated down-regulation of *ABCA1* mRNA, protein and cholesterol efflux was associated with the inhibition of 24(S), 25-epoxycholesterol synthesis (Wong *et al*, 2004). Several reports have shown that synthetic LXR agonists up-regulate *ABCA1* expression, inducing cholesterol efflux and reverse cholesterol transport (Sparrow *et al*, 2002). Agonists of PPAR have also been shown to up-regulate *ABCA1* expression by increasing LXRα expression (Chawla *et al*, 2001).

Our results show a repression of *ABCA1* after cholesterol treatment, only in Caco-2 cells, which is not in agreement with the well known induction mediated by LXR. In the intestine, due to low expression of LXR, other regulatory mechanisms may be in play.

In HepG2 cells, *ABCB11* levels were induced after 4h of treatment decreasing at longer periods. In Caco-2 cells (20%FBS) *ABCB11* mRNA expression was increased after 24h of cholesterol treatment (Results - Paper V).

Cholestasis induced by the hepatotoxicity of drugs may be a consequence of the direct induction of BSEP (Stieger *et al*, 2007). In fact, *ABCB11* mRNA expression is induced when hepatocyte bile acids are elevated or under certain cholestatic conditions (Wolters *et al*, 2002; Zollner *et al*, 2003). In the canalicular membrane, the BSEP expression is induced by FXR, which is the driving force for the formation of bile and is also affected by SHP (Chiang, 2009). The induction of *ABCB11* is dependent of the type of bile acids involved and correlates with the FXR ligand specificity (Parks *et al*, 1999).

Hoek *et al*, described that in the presence of 9-cis-retinoic acid (a derivative of vitamin A, whose malabsorption is associated with cholestasis) chenodeoxycholic acid induces *ABCB11* via FXR/RXR, being this induction even higher in the absence of this vitamin A derivative (Hoek *et al*, 2009). Vitamin D receptor (VDR) has the opposite effect inhibiting *ABCB11* levels through interaction with FXR (Honjo *et al*, 2006).

Besides transcriptional regulation, BSEP seems to be regulated at the post-transcriptional level (Stieger *et al*, 2007). This short-term regulation enables the hepatocyte to modulate bile acid secretion in response to bile acid flux and to pathophysiological conditions (Anwer, 2004).

From our results, an association between excess of cholesterol and the induction of *ABCB11*, probably via FXR, suggests an important role for *ABCB11*, in cholesterol regulation, in liver and intestine.

In HepG2 cells, *ABCC2* was increased after 12h of treatment in response to cholesterol and no significant changes were observed in Caco-2 cells (Results - Paper V).

ABCC2 plays a functional role in the intestinal secretion of many drugs, mediating drug detoxification and limiting the oral absorption of their ligands by extruding them back into the intestinal lumen. This protein also plays a role in reducing the oral availability and biliary and intestinal excretion of the food-derived carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), increasing the plasma value of PhIP (Dietrich *et al*, 2001). ABCC2 is not constitutively expressed and its expression is regulated at transcriptional and post-transcriptional level in response to many compounds and to different disease status. SHP is involved in the regulation of *ABCC2* (Chiang, 2009). Changes in intracellular concentrations of bile acids and other compounds that are substrates of ABCC2 regulate the transcription of *ABCC2* gene, via FXR, PXR or CAR (Kast *et al*, 2002). Feeding mice with bile acids resulted in the induction of *Abcc2* mRNA and protein expression, which may prevent the accumulation of potentially toxic bile acid conjugates or xenobiotics (Fickert *et al*, 2001).

ABCC2 was also induced in the intestine of humans after treatment with rifampicin, a PXR ligand (Fromm *et al*, 2000). In humans, some studies observed a reduction in *ABCC2* mRNA levels in livers from patients with primary sclerosing cholangitis or with obstructive cholestasis (Oswald *et al*, 2001; Shoda et al, 2001). The amount of ABCC2 molecules can also be regulated by a change in synthesis or degradation rate, since obstructive cholestasis leads to reduced ABCC2 protein levels in the intestine without an effect on *ABCC2* mRNA expression (Dietrich *et al*, 2004). This difference has been shown to be due to a more rapid degradation of the transporter in cholestasis than under normal conditions (Paulusma *et al*, 2000).

In a recent study published by Rodrigues *et al*, *ABCC2* and *ABCG2* mRNA levels were increased in patients with hypercholesterolemia compared with normolipidemic individuals while after statin (atorvastatin) therapy gene expression was down-regulated (Rodrigues *et al*, 2009).

The increase of *ABCC2* in hypercholesterolemia is in agreement with the induction we observed for this gene in HepG2 after cholesterol treatment, which indicate that ABCC2 play a role in cholesterol regulation. The mechanisms underlying this induction remain however to be determined.

The levels of *ABCC3* didn't suffer significant changes, in response to cholesterol treatment in both cell lines (Results - Paper V).

ABCC3 shares an overlap in substrate specificity with ABCC2. The affinity of bile acids together with its pattern of expression in the intestine, suggests a possible role for ABCC3 in bile salt reabsorption, as part of the enterohepatic recirculation, probably to function as a backup detoxifying pathway for hepatocytes when normal canalicular route is damaged by cholestatic diseases and the function of ABCC2 is impaired (Oostendorp *et al*, 2009). The levels of *ABCC3* are increased under conditions that result in cholestasis, while *ABCC2* decreases, which may indicate that these two transporters protect liver from accumulation of potentially toxic bile

constituents (Konig *et al*, 1999). Little is published about *ABCC3* regulation, but it was reported that PXR and CAR up-regulate *ABCC3* expression as well as LRH-1, whose response is mediated by bile acids (Xu *et al*, 2005; Inokuchi, 2001). Our results are in agreement with the possible backup function played by ABCC3, since *ABCC2* was induced in the conditions of the experiment. The molecular mechanisms underlying this reciprocal regulation of *ABCC2* and *ABCC3* are not presently clear nor are the potential transcription factors binding differentially to the regulatory region of each gene.

In HepG2 cells the levels of *ABCC6* were decreased after 24h of cholesterol treatment, while in Caco-2 cells (20%FBS) after a slight early decrease, an increase in *ABCC6* mRNA was observed after 24 of cholesterol treatment (Results - Paper V).
ABCC6 is expressed mainly in liver and kidney, but also in intestine (Oostendorp *et al*, 2009). The exact physiological function and substrate specificity of ABCC6 are currently unknown. ABCC6 can transport several cytotoxic compounds that are also substrates of other ABCC transporters (Belinsky *et al*, 2002). The expression of this transporter is low in duodenum and colon (Kool *et al*, 1999a).
Our results point to a role of *ABCC6* in cholesterol regulation, since this gene was responsive in both cell lines, suggesting the involvement in the efflux of cholesterol or cholesterol metabolites. Particularly interesting is the induction observed in HepG2, and the late effect indicates that some degree of metabolism is needed before an induction is observed, suggesting that it is not a direct effect of cholesterol, but more likely a metabolite.

In HepG2 and Caco-2 cells, the levels of *ABCG2* remain unchanged after treatment with cholesterol (Results - Paper V).
ABCG2 is a half transporter expressed at the apical membrane of the small intestine, where it limits drug absorption and facilitates secretion of clinically important drugs back into gut lumen (Maliepaard *et al*, 2001). Induction of *ABCG2* was associated with AhR, E2-related factor (Nrf2) and PXR (Jigorel *et al*, 2006) and the activity of this transporter was reported to be inhibited by membrane cholesterol depletion (Troost *et al*, 2004). In a study by Telbisz *et al*, cholesterol modulated the ATPase activity of ABCG2 in mammalian cells (Telbisz *et al*, 2007). This may indicate that cholesterol might modulate the function of AGCG2 by interaction with membrane domains of the transporter and/or the nucleotide binding domains and might also influence the binding of lipophilic substrates (Romsicki and Sharom, 2001).
A regulation by cholesterol at the level of gene expression has not been described; in our hands, the expression of this is gene was non responsive in both cell lines. An effect on activity as observed by Telbisz et al, can not be ruled out, since the ATPase activity was not determined in these experiments. Our results suggest that this efflux transporter is not likely to contribute to the control of cholesterol homeostasis.

ABCG5 levels were increased in HepG2 cells after 4h and 8h of cholesterol treatment, while in Caco-2 cells (20%FBS) this increase was observed after 8h and 24h of treatment. In Hep2 cells the *ABCG8* mRNA expression was increased at 2h, 4h and 8h after treatment with cholesterol, while in Caco-2 cells (1%FBS) the levels of *ABCG8* increased after 12h of treatment (Results - Paper V).

ABCG5 and ABCG8 transporters promote biliary cholesterol secretion and reduce the absorption of dietary cholesterol (Yu *et al*, 2002). LXR/RXR induces transcription of *ABCG5* and *ABCG8*, which act in the intracellular transport of cholesterol (Tontonoz *et al*, 2003). Mice deficient in *Abcg5/g8* have a reduced biliary cholesterol secretion and enhanced phytosterol absorption but only minimal effects on the efficiency of cholesterol absorption (Yu *et al*, 2002a). However, in hamster or mice, the addition of cholesterol to the diet resulted in an increase of ABCG5 and ABCG8 in liver and small intestine, probably mediated by LXR-mediated pathway, which in turns is associated with increase in biliary sterol secretion (Gylling and Miettinen, 1997). Therefore, under certain conditions ABCG5/G8 seem to play a key role in the control of cholesterol absorption, since the pharmacological induction or overexpression of *Abcg5/g8* in mice results in a reduction in the percentage of cholesterol absorbed from the intestine (Yu *et al*, 2002a). Deoxycholic acid represses *ABCG5* and *ABCG8* promoters, which is consistent with bile acid regulation of these genes via FXR-SHP-LRH-1 pathway (Freeman *et al*, 2004).

There is also a possibility for a post-transcriptional ABCG5/G8 regulation (Kidambi and Patel, 2008).

The induction observed for *ABCG5* and *ABCG8* in HepG2 or in Caco-2 cells after cholesterol treatment is therefore in agreement with the increased expression observed *in vivo* in animal models, in liver and intestine, suggesting an important role for these transporters in cholesterol homeostasis and perhaps potential targets for therapeutic drugs. The mechanisms underlying the observed induction are yet to be established; particularly interesting is the different timing of induction, with a later induction in Caco-2 than in HepG2. This may reflect metabolic differences leading to different concentrations of nuclear receptor ligands along the treatment period, or differential interaction with tissue-specific transcription factors in the regulatory regions.

Our observations for the efflux transporters suggest an important role played by *ABCA1, ABCB11, ABCC2, ABCC6, ABCG5* and *ABCG8* in liver and intestine, since these genes were responsive in either HepG2 or Caco-2, or both, in response to cholesterol treatment, suggesting the existence of a broad response of efflux mechanisms to a common signal, either cholesterol or a metabolite. Also, this cellular response suggests that the activity of these transporters may be important for the maintenance of cholesterol homeostasis.

Taken together, the results obtained in the present work describe a variety of effects of cholesterol in metabolism and transport in liver and intestine cells. Our observations point, in general, to an increased expression of uptake and efflux transporters as well as drug

metabolizing enzymes triggered by cholesterol. However, the majority of the studied genes are known to be repressed by bile acids. Two hypotheses may then be postulated:
a) Cholesterol acts *in vivo* by regulating these genes via conversion into bile acids. This would account for the differences, since in rat liver, almost all cholesterol is metabolized to bile acids, which at high levels in the cell can repress target genes and in HepG2 cells the metabolism of cholesterol may be reduced and bile acids may be present at low levels;
b) Cholesterol mediated changes might occur at transcriptional level via metabolites of cholesterol or even cholesterol itself, which may activate nuclear receptors that regulate the expression of uptake and efflux transporters and drug metabolizing enzymes. Oxysterols are bile acids precursors and they can bind nuclear receptors, such as FXR, LXR, PXR and CAR activating the regulatory mechanisms that assures maintenance of cellular bile acids balance.

We can hypothesize that, for each of the genes analysed, a different scenario will be occurring, depending on the DNA responsive elements present in the regulatory regions, that are putative binding sites for these transcription factors, and on the array of ligand activated and tissue-specific transcription factors present in the liver or intestine cells.

The information gathered in the course of this work opens interesting new perspectives on the crosstalk between the homeostasis of endogenous compounds and the response to drug treatment. Several questions were raised regarding the molecular mechanisms of regulation that now need to be elucidated, with the dissection of the events that in each case are responsible for the observed effect.

Taken together, the results obtained in this work should contribute to a better understanding of the molecular mechanisms underlying the susceptibility of a given individual to disorders in cholesterol homeostasis and the effect of pathologies in drug response. This knowledge should provide important clues for future improved surveillance and therapeutic strategies in the field of cardiovascular diseases.

Chapter V - Conclusions and Future Perspectives

In this work we intended to study pharmacogenetics and expression patterns of the metabolizing enzymes and transporters, identified to play key roles in cardiovascular diseases.

This work is part of a global interest on the role of drug metabolizing enzymes and transporters in disease susceptibility and as determinants of therapeutic success, which has been pursued both at the level of pharmacogenetic determinants and at the level of regulation by xenobiotics. Several genes key to drug metabolism and transport have been analyzed in populations from different ethnic backgrounds, from Portugal, Latin America and Portuguese speaking Africa.
This work went beyond genetic determinants and focused on endogenous molecules as possible regulators of drug metabolism and excretion.

1. Pharmacogenetics of genes candidate to play a role in cardiovascular diseases and therapeutics

Polymorphisms and mutations in genes involved in the transport or metabolism of some therapeutic drugs and that alter the activity of the proteins involved may lead to liver toxicity (decreased activity) or a therapeutic inefficacy (increased activity). Pharmacogenetic studies have an extreme importance since they open the door to a new way of treatment, the personalized therapy. Knowing the genetic background of a population allows developing new, more effective and safe therapeutics for each person. An evaluation of SNP frequencies among different populations with variable ethnic background is certainly useful to characterize the change in drug pharmacokinetics and predict differential susceptibility to possible adverse effects. This assumption led us to characterize some genetic variants, whose genes were more responsive to cholesterol changes, in different ethnic backgrounds. PCR-RFLP genotyping methods were developed for metabolizing enzymes (CYP7A1- A-203C, C-278A, C-496T, A698G, G1039A; CYP7B1- C-116G, C-1774T, G971A; CYP27A1- C490T, C506T, G817A, T1633C), and for influx (SLCO1B1- T217C, A388G, C463A, T521C; SLCO1B3- T334G, G699A, G1564T; SLC22A1- C480G, C886G, T4215C; SLC10A1- C800T, A2587G, A2192G) and efflux (ABCB11- T580C, G779A, C1331T, A2155G; ABCG5- G80C, C148T, C1550G, C1810G; ABCG8- G55C, A161G, C1199A, T1895C) transporters.
Some of these polymorphisms were first described in this study and, although some variants seemed to be highly conserved, the majority of the polymorphisms analysed have similar distributions between Portuguese and Colombian population but significant differences when compared with the Mozambican population. These results show a variable ethnic-background between the analysed populations and the allelic frequencies obtained might result from natural selection in some geographic locations, which might have played a role in diversity at same loci.

2. Expression profile of genes involved in drug metabolism and transport in response to cholesterol

During the past decade there was an increased awareness of the critical interplay between drug metabolizing enzymes and transporters as determinants of drug disposition and response. Several drug metabolizing enzymes and transporters are able to act on both endogenous and exogenous substrates, constituting a potential focus of interface between specific physiological and pathological states and the clearance of xenobiotic substrates such as therapeutic drugs. This interplay prompted us to investigate the changes that occur in genes involved in cholesterol metabolism, not only drug metabolizing enzymes (CYP3A4, CYP7A1, CYP27A1, CYP39A1, CYP51A1, CYP7B1, CYP8B1), but also influx (SLC10A1 (NTCP), SLC22A1 (OCT-1), SLC22A7 (OAT2), SLCO1A2 (OATP-A), SLCO1B1 (OATP-C), SLCO1B3 (OATP-8), SLCO2B1 (OATP-B)) and efflux (ABCA1, ABCB11 (BSEP), ABCC2 (MRP2), ABCC3 (MRP3), ABCC6 (MRP6), ABCG2 (BCRP), ABCG5, ABCG8) transporters.

The majority of the genes studied responded to changes in cholesterol and, in some cases, differently between cellular models. Alterations in the functional characteristics and/or expression levels of drug transporters and drug metabolizing enzymes in the liver and intestine may conceivably contribute to the variability in drug oral bioavailability often observed in the clinical settings.

The knowledge related with the expression pattern of drug transporters and drug metabolizing enzymes in tumour cells may help do develop target drugs to specific tumours. The best target transporters and enzymes are the higher expressed and/ or up-regulated in tumour cell lines. Drug toxicity in specific organs may also be prevented since different combination of transporters or drug metabolizing enzymes are responsible for drug transport and metabolism in tumour cell and for a specific toxic effect, that can be prevented by co-medication with a drug that can inhibit the transport or metabolism of the drug acting in the tumour cell but without influencing the tumour treatment. This work contributes for this knowledge, since we characterized the expression pattern levels of uptake transporters, drug metabolizing enzymes and efflux transporters important not only in cardiovascular diseases but that can also play a key role in tumours.

The expected results should contribute to a better understanding of the molecular mechanisms underlying the susceptibility of a given individual to disorders in cholesterol homeostasis. This knowledge should provide important clues for future improved surveillance in the field of cardiovascular diseases.

3. Future Perspectives

The effects of polymorphisms and epigenetic alterations on gene expression should be more explored. When levels of pharmacogenetic diversity are high, as in this study, this genetic analysis assumes higher importance, because the efficacy of treatment can depend on it. The elucidation of the molecular mechanisms and the way polymorphisms can affect activity of enzymes and transporters involved in cholesterol and bile acids metabolism should help to found new therapeutic compounds, more efficient and safe with dosage adjusted to the genetic profile of each person. This is the objective of pharmacogenetics, optimize drug treatment, with drug selection and drug dosage adapted to the patient's genetic background. To reach that objective, it is necessary an intensive study of genetic variants and how they affect the metabolic pathways, in which proteins are involved. In addition, the genetic analysis of individuals from different geographic locations is also necessary, to establish the differences existing between genetic patterns, which are ethnic-dependent.

In future genetic analysis one point to consider is the sample size, which should be large enough to guarantee statistical power.

Drug metabolizing enzymes together with influx and efflux transporters are important regulators of the flux of bile acids in the enterohepatic circulation and they have the possibility to modulate bile acid signalling and metabolic regulation. Therefore, it will be important to further understand the regulation of these enzymes and transporters and their relationship to cholesterol and bile acids metabolites as well as in human disease. It should be also important to understand how therapeutic compounds interact with these enzymes and transporters and how they affect the bile acids transporters in animal models and patients.

The genes more responsive to cholesterol should be studied at molecular level and the molecular mechanism of action of a selected agent/regulator should also be studied through characterization of DNA/protein interactions (e.g. by gel mobility shift assay).

This task will allow characterize the molecular mechanisms underlying cholesterol and bile acids' effect on gene expression.

Chapter VI - References

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Appendix I

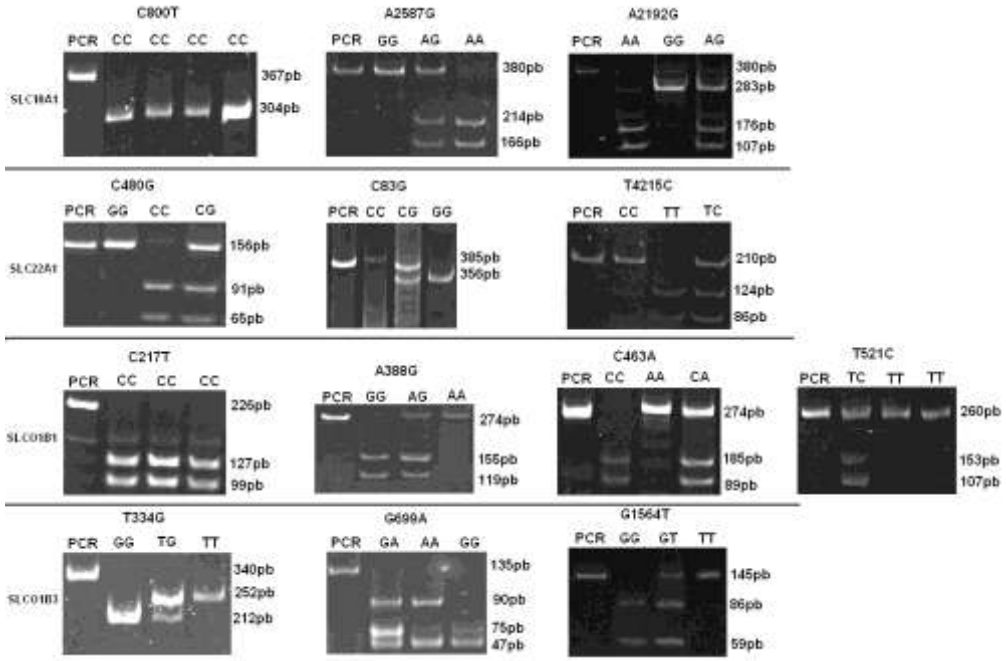

Figure 1: SLC10A1, SLC22A1, SLCO1B1 and SLCO1B3 Polymorphisms. Patterns of DNA fragments obtained for each genotype identified.PCR-RFLP analysis was performed using specific primers and enzymes described in Table 1 and Table 2 (Results – Paper I)

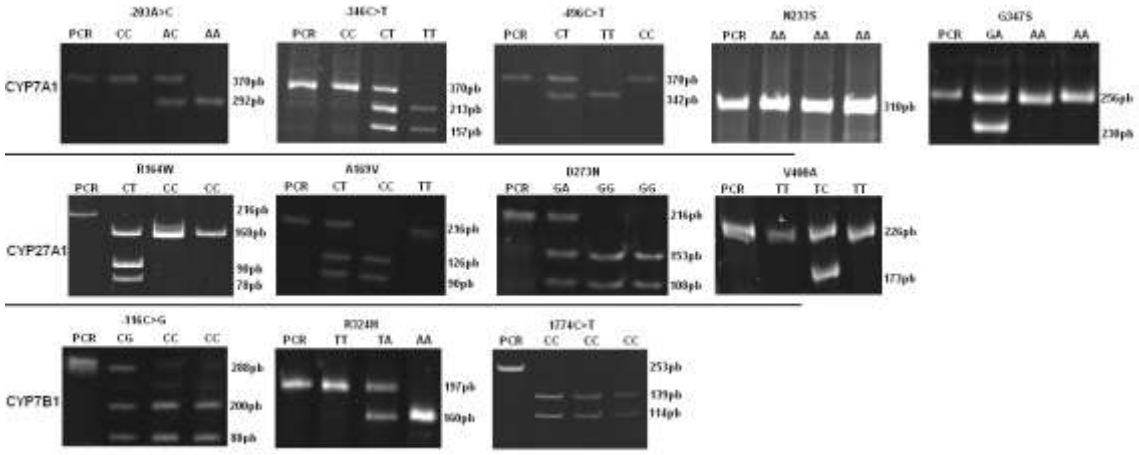

Figure 2: CYP7A1, CYP27A1 and CYP7B1 Polymorphisms. Patterns of DNA fragments obtained for each genotype identified.PCR-RFLP analysis was performed using specific primers and enzymes described in Table 1 and Table 2 (Results – Paper II)

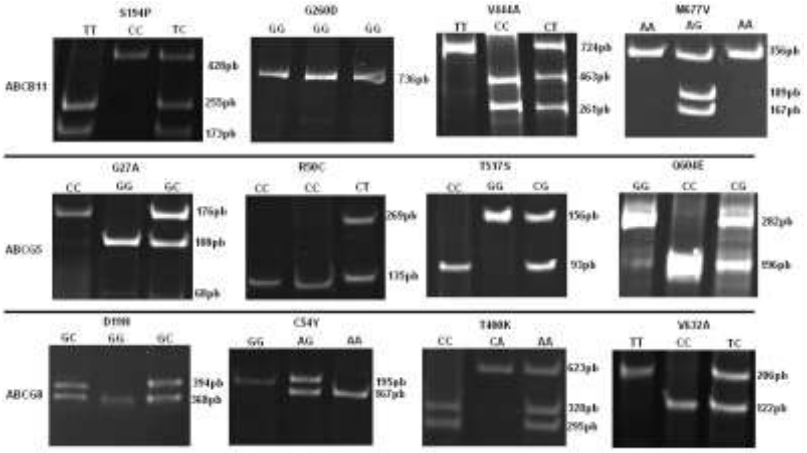

Figure 3: ABCB11, ABCG5 and ABCG8 Polymorphisms. Patterns of DNA fragments obtained for each genotype identified.PCR-RFLP analysis was performed using specific primers and enzymes described in Table 1 and Table 2 (Results – Paper III)

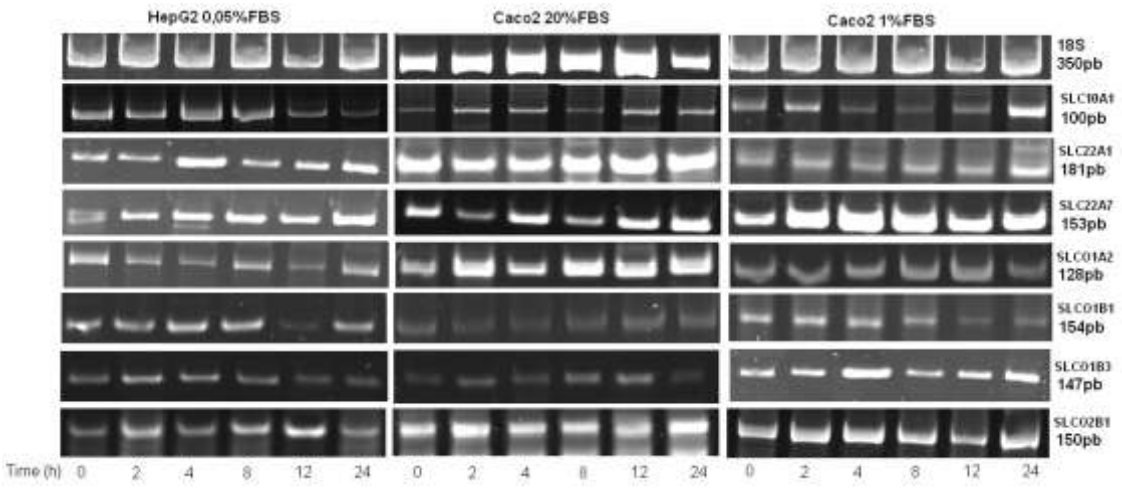

Figure 4: Expression of SLC transporters in HepG2 and Caco-2 cell lines. Cell culture media was supplemented with 5µg/ml cholesterol for different periods as indicated. SLC mRNA relative levels were assessed by RT-PCR analysis using specific primers as described in Table 1 (Results - Paper V)

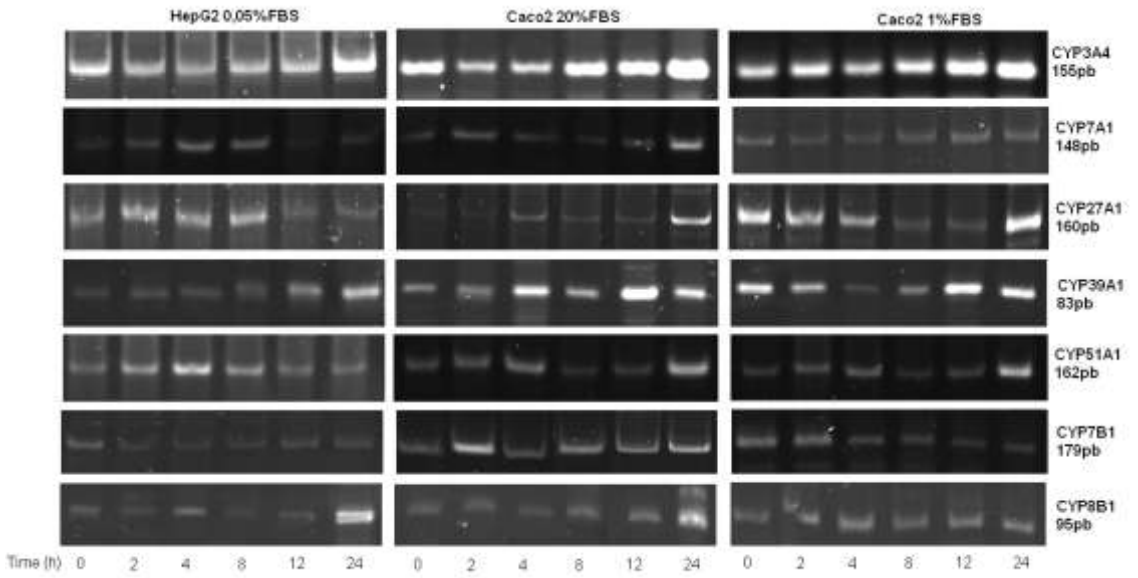

Figure 5: Expression of CYP enzymes in HepG2 and Caco-2 cell lines. Cell culture media was supplemented with 5µg/ml cholesterol for different periods as indicated. CYP mRNA relative levels were assessed by RT-PCR analysis using specific primers as described in Table 1 (Results - Paper V)

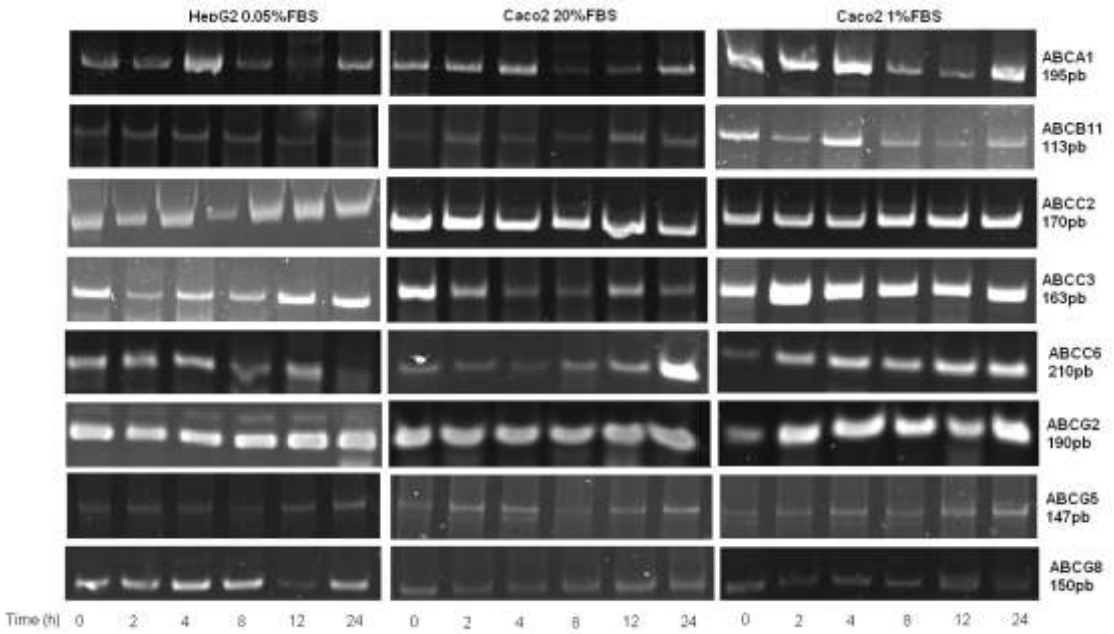

Figure 6: Expression of ABC transporters in HepG2 and Caco-2 cell lines. Cell culture media was supplemented with 5µg/ml cholesterol for different periods as indicated. ABC mRNA relative levels were assessed by RT-PCR analysis using specific primers as described in Table 1 (Results - Paper V)