Studies on the mechanism of regulation of bile acid synthesis in humans with some aspects on genetic factors

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ABSTRACT

In the present investigation we studied human bile acid synthesis and its regulation both in vivo and in vitro and also evaluated a possible influence of polymorphisms in the CYP7A1 and CYP8B1 genes on bile acid synthesis in vivo. The following observations were made:

• Primary human hepatocytes cultured on matrigel under serum-free conditions produced bile acids consistent with the pattern in vivo, i.e. predominately CA (70%) and CDCA (25%), both conjugated with glycine or taurine.

• Both glycine-conjugated and free bile acids suppressed bile acid synthesis and mRNA levels of CYP7A1 in cultures of human primary hepatocytes in the order CDCA>DCA>CA>UDCA. Also mRNA levels of CYP8B1 and CYP27A1 were suppressed but to a much lesser extent. Addition of GCDCA as well as GDCA resulted in higher levels of mRNA for SHP supporting a mechanism by which FXR suppresses CYP7A1 through induction of SHP.

• Upregulation of bile acid synthesis in humans by cholestyramine treatment resulted in higher levels of mRNA of not only CYP7A1, but also of HNF-4α. This is consistent with a mechanism in which HNF-4α is an important stimulating nuclear factor involved in bile acid synthesis.

• Earlier described polymorphisms in the promoter region of the CYP7A1 gene could not be associated with variation in CYP7A1 enzyme activity in liver biopsies or rate of bile acid synthesis evaluated by serum levels of 7α-hydroxy-4-cholesten-3-one or by isotope dilution kinetics. The genotypes did not differ in experiments with a reporter system for transcriptional activity or in EMSA analysis measuring binding of nuclear extracts.

• Differences in ratio of CA and CDCA in gallbladder bile observed in a number of human subjects could not be explained by polymorphisms in the CYP8B1 gene.

The results obtained emphasize marked species differences in the mechanisms for regulation of bile acid synthesis. Genetic polymorphism in the key enzymes does not appear to be of major importance for this regulation.
This thesis is based on the following publications

I. Bile acid synthesis in primary cultures of rat and human hepatocytes.
Ellis E, Goodwin B, Abrahamsson A, Liddle C, Mode A, Rudling M, Björkhem I, and Einarsson C.

II. Feedback regulation of bile acid synthesis in primary human hepatocytes: evidence that CDCA is the strongest inhibitor.
Hepatology 2003;38:930-938.

Abrahamsson A, Gustafsson U, Ellis E, Nilsson L-M, Sahlin S, Björkhem I, and Einarsson C.
Biochem Biophys Res Commun 2005May6;330(2)395-399.

IV. Common polymorphisms in the CYP7A1 gene do not contribute to variation in rates of bile acid synthesis and plasma LDL cholesterol concentration.
Atherosclerosis 2005Sep;182(1):37-45.

V. Polymorphism in the coding part of the sterol 12α-hydroxylase gene does not explain the marked differences in the ratio of cholic acid and chenodeoxycholic acid in human bile.
Abrahamsson A, Gåfvels M, Reihnér E, Björkhem I, Einarsson C, and Eggertsen G.
CONTENTS

1 INTRODUCTION 8
1.1 Enterohepatic circulation 8
1.2 Cholesterol balance 9
1.3 Bile acid synthesis 9
1.3.1 The neutral pathway 10
1.3.2 The acidic pathway 11
1.4 Regulation of bile acid synthesis 12
1.4.1 Transcriptional factors and nuclear receptors 13
1.4.2 Regulation of CYP7A1 13
1.4.2.1 SHP-dependent pathway 14
1.4.2.2 SHP-independent pathway 15
1.4.3 Regulation of CYP8B1 16
1.4.4 Regulation of CYP27A1 16
1.4.5 Regulation of CYP7B1 17
1.5 Inborn errors in bile acid synthesis and genetic polymorphism 19
1.5.1 CYP7A1 deficiency 19
1.5.2 CYP8B1 deficiency 20
1.5.3 CYP27A1 deficiency 20
1.5.4 CYP7B1 deficiency 21
1.5.5 CYP7A1 polymorphism 21
1.6 In vivo and in vitro models for studies on bile acid synthesis 22

2 AIMS OF THE STUDY 24

3 METHODS 25
3.1 Isolation of rat hepatocytes 25
3.2 Isolation of human hepatocytes 25
3.2.1 Washing and plating of the hepatocytes 26
3.3 Preparation of microsomes and assay of CYP7A1 activity 26
3.4 Bile acid analysis 27
3.5 Isolation of tNA in Papers I-II 28
3.6 Solution hybridization of CYP7A1 mRNA 29
3.7 RNase protection assay 29
3.8 Real time PCR 30
3.9 DNA preparation 31
3.10 Single-stranded-conformation polymorphism (SSCP) 31
3.11 Genotyping with restriction fragment length polymorphisms 32
3.12 DNA constructs 32
3.13 Electromobility shift assay (EMSA) 32
3.14 Transient transfection assay 33

3 RESULTS AND CONCLUSIONS 34
4.1 Establishing a cell culture model with human hepatocytes which would be suitable for studies of bile acid synthesis 34
4.1.1 Conclusion 35
4.2 Effect of different bile acids on acid synthesis and key bile
Enzymes and nuclear factors involved in bile acid synthesis
in cultures of primary human hepatocytes
35
4.2.1 Conclusion 36

4.3 Effect of up- and downregulation of bile acid synthesis
in gallstone patients on key enzymes and nuclear factors
involved
36
4.3.1 Conclusion 37

4.4 Screening for unknown polymorphisms and evaluation of
influence of reported polymorphisms in the CYP7A1 gene
on enzyme activity, bile acid synthesis and LDL cholesterol
in serum
38
4.4.1 Conclusion 39

4.5 Screening for polymorphisms in the CYP8B1 gene that
could explain the variability in proportion of CA and CDCA
in gallbladder bile
39
4.5.1 Conclusion 40

5 GENERAL DISCUSSION 41

6 ACKNOWLEDGEMENTS 46

7 REFERENCES 48
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>CA</td>
<td>Cholic acid</td>
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<tr>
<td>CDCA</td>
<td>Chenodeoxycholic acid</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>Cholesterol 7α-hydroxylase</td>
</tr>
<tr>
<td>CYP7B1</td>
<td>Oxysterol 7α-hydroxylase</td>
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<tr>
<td>CYP8B1</td>
<td>Sterol 12α-hydroxylase</td>
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<td>CYP27A1</td>
<td>Sterol 27-hydroxylase</td>
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<td>DCA</td>
<td>Deoxycholic acid</td>
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<tr>
<td>FTF</td>
<td>Alpha fetoprotein transcription factor</td>
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<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
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<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
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<tr>
<td>HNF-4α</td>
<td>Hepatocyte nuclear factor-4α</td>
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<tr>
<td>LCA</td>
<td>Lithocholic acid</td>
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<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
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<tr>
<td>PPARα</td>
<td>Peroxisome proliferation activating receptor α</td>
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<td>PXR</td>
<td>Pregnane X receptor</td>
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<td>RXRα</td>
<td>Retinoic X receptor α</td>
</tr>
<tr>
<td>SHP</td>
<td>Small heterodimer partner</td>
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<tr>
<td>UDCA</td>
<td>Ursodeoxycholic acid</td>
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1 INTRODUCTION

1.1 ENTEROHEPATIC CIRCULATION

Bile acids are synthesized in the hepatocytes. In humans two primary bile acids are formed; cholic acid (CA) and chenodeoxycholic acid (CDCA) [1-3]. They are conjugated with an amino acid, in humans glycine or taurine, which makes them more soluble in water. They are secreted into the bile ducts and transported to the gallbladder where they are stored for further excretion into the small intestine when the gallbladder is contracted, stimulated by a meal. In the small intestine the bile mixes with the meal and the bile acids form mixed micelles with the lipids in the intestine. The bile acids act as detergents and emulsify the lipids in the food, i.e. cholesterol, fat-soluble vitamins and fatty acids.

In the distal part of the small intestine the bile acids are partly deconjugated and dehydroxylated by bacterial enzymes. The dehydroxylation of the primary bile acids results in the so called secondary bile acids which in humans are deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) [1-3].

The bile acids are reabsorbed by the small intestine predominantly by the distal ileum via an active transporter called intestinal bile acid transporter (IBAT) [4]. The dihydroxy bile acids are also absorbed in the proximal part of the small intestine resulting in a faster return of these bile acids to the liver. In this way about 95-98% of the bile acids are transported back to the liver by the portal vein and rapidly extracted by the hepatocytes to about 70-90%. The remaining bile acids reach the general circulation and return to the liver via the hepatic artery. The bile acids are able to regulate their own synthesis by means of negative feedback.
1.2 CHOLESTEROL BALANCE

Cholesterol is an essential compound in the body. It is the precursor for several steroid hormones and is an important structural compound in membranes. The body can both synthesize cholesterol as well as utilise the cholesterol that is supplied through the food. Cholesterol is mainly synthesized in the liver and can also be stored there in esterified form. It can also be transported in lipoproteins to other organs in the body by the blood circulation. It can be incorporated in the tissue and used for structural maintenance or be stored in the receiving cell. Cholesterol can also be transported back to the liver by HDL-lipoproteins (the “good” cholesterol). In the steady state the received and returned cholesterol should be about the same [5]. If not, the cell may become overloaded with cholesterol resulting in changed cell structure. This may lead to diseases such as atherosclerosis [6]. The main pathway for the body to eliminate cholesterol is through the bile where it can be secreted both directly as free cholesterol or after conversion into bile acids.

1.3 BILE ACID SYNTHESIS

In the human liver about 500 mg of cholesterol is converted into bile acids daily.

Bile acid synthesis requires enzymes present in several different cell organelles: endoplasmatic reticulum, both smooth and rough, cytosol, mitochondria and peroxisomes. Traditionally, the bile acid synthesis is following two major pathways: the neutral (classical) and the acidic (alternative) pathway (Figure 1). Acidic sterols are early intermediates in the acid pathway whereas neutral sterols are early intermediates in the neutral pathway [7]. In
contrast to the neutral pathway, the first steps in the acidic pathway may occur extrahepatically.

**Figure 1.** Major pathways in bile acid synthesis.

### 1.3.1 THE NEUTRAL PATHWAY

The neutral pathway starts with a 7α-hydroxylation of the steroid nucleus catalysed by the cholesterol 7α–hydroxylase (CYP7A1), the rate limiting enzyme in this pathway. Newly synthesized cholesterol is the preferred substrate for this enzyme. In the next step, 7α-hydroxycholesterol is converted into 7α-hydroxy-4-cholesten-3-one [8]. Both 7α-
hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one can be measured in serum and may serve as indicators of bile acid synthesis [9, 10]. After these two initial reactions, 7α-hydroxy-4-cholesten-3-one may be hydroxylated in the 12α-position by the sterol 12α-hydroxylase (CYP8B1) [11, 12] followed by its further transformation into CA. If 7α-hydroxy-4-cholesten-3-one is not hydroxylated in the 12α-position the end product will be CDCA rather than CA. All the above reactions take place in the microsomal compartment.

After these initial steps the modified sterol is further processed by the sterol 27-hydroxylase (CYP27A1) located in the mitochondria. This enzyme introduces a hydroxyl group in 27-position (side chain of the sterol compound). The side chain undergoes further oxidation by either CYP27A1 or by alcohol and aldehyde dehydrogenases present in the cytosol. To form the final products the intermediate C27-acids undergo β-oxidation and side-chain cleavage in the peroxisomes.

1.3.2 THE ACIDIC PATHWAY

The acidic pathway starts with oxidation of the cholesterol side chain by CYP27A1 in the mitochondria. After this initial step, the 27-hydroxycholesterol formed can either have its side chain shortened in the same manner as in the neutral pathway, or undergo a 7α-hydroxylation catalysed by the oxysterol 7α-hydroxylase (CYP7B1) [13, 14]. After the 7α-hydroxylation step the steroid nucleus is modified in a similar way as in the neutral pathway. The intermediates from the acidic pathway are less prone to being 12α-hydroxylated by CYP8B1 and hence the main product of this pathway is CDCA.

In the normal human liver the neutral pathway is believed to dominate over the acidic one.
In other species, several other bile acids are formed. Among these are ursodeoxycholic acid (UDCA), which has received its name from being found in bear bile, and α- and β-muricholic acid which are found in the mouse and rat.

1.4 REGULATION OF BILE ACID SYNTHESIS

As mentioned above, bile acid synthesis is regulated by feedback inhibition performed by the bile acids returning to the liver through the enterohepatic circulation. After bile fistulation of experimental animals or humans the returning concentrations of bile acids in the portal vein drop to almost zero and the feedback loop is interrupted. The bile acid synthesis is upregulated to compensate for the bile acids lost in the fistula [15]. The same result is achieved by feeding with the bile acid sequestrant, cholestyramine (Questran®). This compound binds bile acids in the small intestine resulting in reduced reabsorption of the bile acids. mRNA levels for CYP7A1, activity levels of the enzyme in liver biopsies and serum levels of 7α-hydroxy cholesterol and 7α-hydroxy-4-cholesten-3-one increase, all indicating a higher rate of synthesis [9, 10, 16, 17].

Treatment of a subject with a bile acid, e.g. CDCA, causes a repressed synthesis of bile acids [18]. In this case the hepatocyte is exposed to higher concentrations of bile acids than normally and the synthesis is turned down to a minimum. In liver biopsies the activity of CYP7A1 and the mRNA levels for the enzyme drop in parallel with circulating levels of intermediates such as 7α-hydroxy-4-cholesten-3-one and 7α-hydroxycholesterol [16, 17, 19, 20].

Earlier research has shown that different bile acids have different capacity to suppress bile acid synthesis in oral loading experiments in humans [18, 21-24]. The suppressive capacity of
an individual bile acid was found to correlate with its hydrophobicity index, and this correlation was for a long period thought to be the explanation for the difference in effect [25]. There is an important species difference in the capacity of different bile acids to suppress the synthesis. CDCA is thus the most effective suppressor in man[18], whereas CA is the most effective in mice [26].

1.4.1 TRANSCRIPTIONAL FACTORS AND NUCLEAR RECEPTORS

How this negative feedback is achieved in the nucleus of the hepatocyte has been elucidated during recent years. Many of the transcription factors involved in the regulation of bile acid synthesis are nuclear receptors.

1.4.2 REGULATION OF CYP7A1

CYP7A1 is an enzyme only present in the liver. CYP7A1 shows a circadian rhythm with varying activity during night and day [27]. Some studies have shown that thyroid hormone and corticosteroids stimulate the activity of CYP7A1. However also negative responsive elements for corticosteroids and thyroid hormone have been found in the promoter of the human gene [28]. In hyperthyroid patients bile acid synthesis is somewhat altered compared to euthyroid subjects. The synthesis of CA is lower suggesting an inhibition of CYP8B1 but the overall rate of bile acid synthesis is not altered [29, 30]. In the intact rat, on the other hand, the two hormones appear to have additave stimulatory effects on CYP7A1 [31]. In hypophysectomized rats substitution with growth hormone but not corticosteroids or thyroid hormone normalised bile acid synthesis [32]. In rat liver thyroid hormone suppressed Cyp8b1
activity but induced Cyp7a1 [33, 34]. This, amongst a lot of other results, indicate that there are important species differences in the regulation mechanisms of bile acid synthesis. It is evident that findings in for example murine species cannot automatically be transferred to the human situation.

The human CYP7A1 gene is located in chromosome 8 q11-q12 and contains 6 exons and 5 introns [35]. The protein is a single polypeptide chain of 500 amino acids and is highly conserved.

In the promoter region of the CYP7A1 gene two potential nuclear factor binding areas have been identified. These areas, called bile acid responsive element (BARE) I and II, are highly conserved [36]. Marked species differences have been documented in the ability to bind different nuclear factors in these areas. For instance in the mouse BARE I there is a binding site for LXRα [37] which activates the gene, thereby promoting the conversion of excess cholesterol into bile acids (Figure 2). In the human gene there is no such binding site which explains why bile acid synthesis is induced by cholesterol in mice but not in humans. The BARE I also contains a binding site for the pregnane X receptor (PXR). The BARE II area contains both binding areas for hepatocyte nuclear factor-4α (HNF-4α), retinoic acid receptor α (RARα), α-fetoprotein transcription factor (FTF) and farnesoid x receptor (FXR) (Figure 2) [36].

1.4.2.1 SHP-DEPENDENT PATHWAY

The first nuclear receptor found to be involved in the process of regulating bile acid synthesis was FXR [38]. After binding, bile acids are able to activate this receptor [39]. When the different bile acids were compared, CDCA was found to have the highest affinity for the
binding site [40]. This explains the high potency of this specific bile acid to exert negative feedback in humans.

FXR is involved in the regulation of both CYP7A1 and CYP8B1.

FXR then activates the transcription of short heterodimer partner (SHP) by binding to a site in the SHP promoter (Figure 2). SHP is also a nuclear receptor but with an unusual structure lacking a DNA binding domain. SHP binds to FTF and inhibits it [41]. The SHP promoter region also contains a functional FTF element.

FTF, also a nuclear receptor, normally activates the transcription of both the CYP7A1 and CYP8B1 genes. When SHP binds to FTF it is inactivated and the activation of CYP7A1 inhibited. The mRNA levels of CYP7A1 decrease and the activity goes down resulting in less bile acid synthesis. FTF, in its turn, decreases the SHP transcription through its functional element in the SHP promoter and in this way there is an autoregulation of this pathway. SHP may also inhibit the activating action of HNF-4α on the CYP7A1 gene (Figure 2).

**1.4.2.2 SHP-INDEPENDENT PATHWAY**

In SHP knock out mice, where the above mechanism is impaired, bile acids were still found to be able to exert negative feedback on their own synthesis [42]. The mechanism by which the bile acids achieve this is called the SHP independent pathway. This pathway is thought to be initiated via the cJun N-terminus kinase (JNK) pathway. Bile acids can induce inflammatory response in the macrophages of the liver, with cytokine release as a result. These cytokines, TNFα and IL-1β, can cross the sinusoid and enter the hepatocyte where they may inhibit the CYP7A1 expression through inactivation of the HNF-4α stimulatory effect on the same gene. HNF-4α also interacts with peroxisome proliferators-activated receptor γ (PPARγ) coactivator (PGC-1α) leading to induced transcription of the CYP7A1 gene. The interaction can be
interrupted by PXR which effects PGC-1α and hence inhibits the activation of HNF-4α and its effect on the gene (Figure 2). PXR can be induced by lithocholic acid (LCA) and this can also be a mechanism for bile acids to influence their own synthesis. In addition PXR induces CYP3A4 which catalyzes the 6-hydroxylation of bile acids which is important for renal excretion. From a clinical point of view, this mechanism is very interesting because rifampicin has been used for several decades to reduce pruritus in patients with cholestatic liver disease. Rifampicin, a PXR-agonist, reduces bile acid synthesis and facilitates bile acid excretion through the kidneys [43, 44].

### 1.4.3 REGULATION OF CYP8B1

CYP8B1 is specific for the liver and essential for the synthesis of CA. The activity of this enzyme is also effected by bile acids in a similar way as that of CYP7A1. The human gene is located on chromosome 3p21.3-p22 and contains only one exon and no introns [45]. The protein consists of a single polypeptide chain.

FTF and HNF-4α also bind to the promoter of CYP8B1 and effect the gene in a similar way as the CYP7A1 gene (Figure 2) [46]. The enzyme shows circadian rhythm just as CYP7A1.

### 1.4.4 REGULATION OF CYP27A1

CYP27A1 is present in almost all tissue. In humans there seems to be little or no feedback regulation of this enzyme by bile acids [47]. In the promoter region of the gene there is an HNF-4α as well as a FTF binding site (Figure 2), where the binding of FTF is less efficient than the binding of HNF-4α. The latter factor is believed to be most important in the
regulation of this gene [48]. As in the case of CYP7A1 the gene expression can be decreased by cytokines such as TNFα and IL-1β.

### 1.4.5 REGULATION OF CYP7B1

Very little is known about the regulation of CYP7B1. The substrate specificity of this enzyme is broad and it is present in many different tissues. Wu and Chiang showed that the CYP7B1 promoter region contains regions that bind Sp1 proteins which are expressed in many tissues [49]. The Sp1 proteins are involved in many regulatory events such as cell differentiation and cell cycle control. They also interact with sterol binding responsive element (SREBP) and stimulate the genes involved in cholesterol and fatty acid synthesis. There is a sterol responsive element SRE-like site in the human CYP7B1 promoter region, but its function is yet uncertain [50].

In cultures of primary rat hepatocytes Cyp7b1 is repressed by treatment with bile acids as well as by the cholesterol synthesis inhibitor squalestatin. *In vivo*, Cyp7b1 appears to be regulated by bile acids in rats. This possibility has not been tested in humans.

Cyp7b1 in the rat displays a diurnal rhythm much like the rhythm of Cyp7a1, but somewhat delayed. Since Cyp7b1 is not catalyzing a rate-limiting step in bile acid synthesis, the regulatory effects demonstrated are probably less important from a biological point of view.

The relative importance and the effect of up and down regulation of bile acid synthesis of all these nuclear factors have neither been studied *in vivo* in humans nor *in vitro* in primary human hepatocytes.

Figure 2 Summarises the role of the different nuclear factors in the regulation of bile acid synthesis.
Figure 2. Nuclear receptors and factors involved in regulation of bile acid synthesis in humans and in rat. → symbolizes stimulatory/positive influence and inhibitory/negative influence.
1.5 INBORN ERRORS IN BILE ACID SYNTHESIS AND GENETIC POLYMORPHISM

Inherited disorders involving bile acid synthesis have been identified both in humans and in other species. Presence of homozygous dysfunctional enzymes may result in severe phenotypes with impaired cholesterol and bile acid homeostasis.

1.5.1 CYP7A1 DEFICIENCY

Pullinger et al [51] described a family with 3 individuals displaying a homozygous deletion mutation in the gene. The mutation led to a frameshift causing a change in the activity site of the enzyme. Two of three subjects had no CYP7A1 activity when measured in liver biopsies, but surprisingly the third had such activity. The activity was decreased by 70% in this subject. All the subjects had high levels of total and LDL cholesterol in serum and also higher cholesterol content in the liver biopsies. The amounts of bile acids secreted in the stool were considerably decreased, and the activity of CYP27A1 was found to be increased. Two of the subjects were male and both had been operated on for gallstone disease in their early forties, one of them had had coronary by pass surgery. Their sister was not clinically affected by the deficiency.

A mouse model with a disrupted Cyp7a1 gene has been constructed. The phenotype was more severe with early death and high levels of cholesterol in the liver [52]. Cyp27a1 was compensatory upregulated to replace the neutral pathway for bile acid synthesis [53]. The
species difference regarding cholesterol levels in serum associated with CYP7A1 deficiency probably reflect the differences in regulation of bile acid synthesis between the species. The possibility that other mutations in the CYP7A1 gene may explain the great inter-individual variations in CYP7A1 activity that have been reported has not earlier been addressed.

1.5.2 CYP8B1 DEFICIENCY

To our knowledge, CYP8B1 deficiency in humans has not yet been reported in the literature. Impairment of this enzyme would be expected to lead to loss of CA. Probably such a phenotype may have little or no clinical symptoms. However in earlier studies we have noticed marked differences in ratio between CA and CDCA in human bile without correlation to treatment or gallstone disease. No apparent explanation for these differences has been found and the possibility of a polymorphism in the CYP8B1 gene must be considered.

1.5.3 CYP27A1 DEFICIENCY

The CYP27A1 is expressed in many organs in the body. As mentioned above, in contrast to CYP7A1 it can mediate the hydroxylation of sterols in 27, 25 and 24 position. There are more than 40 mutations described that cause deficiency in CYP27A1. The phenotype described associated with this deficiency is called Cerebrotendinous xanthomatosis (CTX) [54]. The patients have reduced synthesis of normal bile acids, and accumulation of cholesterol and cholestanol in different tissues probably due to a reduced capacity to export the non-hydroxylated compounds. The most serious symptoms may come from the brain where there
is an accumulation of sterols in xanthomas causing dysfunction and eventually death. CTX can be treated with orally administered bile acids.

### 1.5.4 CYP7B1 DEFICIENCY

Setchell et al [55] have described a subject with CYP7B1 deficiency. The affected individual was diagnosed with liver failure in infancy because of accumulation of hepatotoxic oxysterols that could not be 7α-hydroxylated as a result of the deficiency. The DNA sequencing data showed a nonsense mutation in exon 5 resulting in a premature truncation of the protein and loss of activity. Bile acid treatment did not affect the clinical course of the disease and the patient was treated with liver transplantation.

### 1.5.5 CYP7A1 POLYMORPHISM

Wang et al [56] and later Couture et al [57] have reported that a common polymorphism in the promoter region of CYP7A1 may be associated with alterations in LDL cholesterol levels in serum. The polymorphisms reported were a C→A substitution in position -203 and a T→C substitution at position -469 where the -203 C allele always is coupled to the -469 T allele. The nomenclature describing these polymorphisms differs due to the definition used for starting numbering: at the transcription or the translation start point. The subjects homozygous for -203C/-469T allele in these studies had significantly higher serum levels of LDL cholesterol. In the report by Wang et al, these polymorphisms were estimated to account for approximately 15% of the variation in LDL cholesterol levels in serum. Kovar et al [58] reported higher LDL cholesterol levels in serum after cholesterol feeding in subjects homozygous for -203C. Recently, Hofman et al also reported not only association of these polymorphisms to LDL cholesterol concentrations in serum but also association to increased
risk of atherosclerosis in the carotid artery and higher relative risk to experience a new clinical event (myocardial infarction, stroke, transient ischemic attack and death) [59]. However, associations between these polymorphisms in the CYP7A1 gene and levels of enzyme activity or bile acid synthesis had not been reported when the present study was initiated.

1.6 IN VIVO AND IN VITRO MODELS FOR STUDIES ON BILE ACID SYNTHESIS IN HUMANS

Studies on human bile acid synthesis and the effects on the different key enzymes require human liver tissues. Our group has studied patients that are scheduled to undergo cholecystectomy for gallstone disease or gallbladder related symptoms [16, 17]. After informed consent, the patients are treated with e.g. cholestyramine or different bile acids in excess to influence the synthesis in vivo. To avoid any diurnal differences the patients are all operated in the morning and liver biopsy is obtained peroperatively. During the last decade an increasing part of gallbladder surgery has been performed laparoscopically which limits the amount of tissue that can safely be obtained from the liver. Through the years the technical equipment and the experience have improved.

As in all in vivo studies the possibility of inter-individual differences has to be considered. To avoid obvious possible problems we exclude all patients that are overweight, hyperlipidemic, medicated in any way, have undergone intestinal resections, have an abnormal thyroid function or abnormal liver function tests. This in vivo approach has its limitations, however. We cannot treat the subjects with high doses of drugs, the subjects may show bad compliance with the treatment, and we cannot obtain liver samples from the same patient both before and after treatment. In vivo it is not possible to identify the effect of an individual bile acid since
the hepatocyte is affected not only by the administered bile acid but rather by a mixture of bile acids where the administered may dominate.

To study the effect of treatment on human bile acid synthesis over time and under less physiological conditions an in vitro system is necessary. In vitro models using cell cultures can be divided into primary cells and cell lines, usually from hepatoma tumours. Studies on bile acid synthesis have been performed on cell lines but there are important differences between the in vivo pattern of bile acid and the pattern produced in the cell lines [60, 61]. The probably most utilised cell line, Hep G2 cells, produce large amounts of bile acid precursors and unconjugated bile acids [62]. The cells are dedifferentiated and do not maintain certain hepatocyte functions such as albumin secretion and cytochrome p450 expression. Cultures of primary human hepatocytes however do maintain these characteristics [63]. To maintain their polarity and cell to cell communication, hepatocytes require attachment to extra cellular basement membranes. These features are important for the hepatocyte phenotype [64]. When hepatocytes are cultured on collagen, they lose the ability to express hepatocyte-specific genes which suggests dedifferentiation. In the microscope it is evident that the hepatocytes change shape and more resemble fibroblasts [65]. When hepatocytes are cultured on matrigel they keep their original appearance and even form bile canaliculi, tight and gap junctions which are important for the cell to cell communication. Since several years human primary hepatocytes are commercially available. The quality and function of the cells differ widely, however. When the present work was initiated, no studies on the regulation of human bile acid synthesis had been performed on primary human hepatocytes.

The need for a cell system that is consistent in its expression of enzymes and resembles the in vivo situation is evident, and would be complementary to the in vivo model.
2  AIMS OF THE STUDY

The purpose of this investigation was to elucidate mechanisms of regulation of bile acid synthesis in humans - both in vitro and in vivo - and to evaluate the influence of genetic factors such as polymorphisms in the genes of key enzymes. The following subprojects were defined:

1. To establish a cell culture model with human primary hepatocytes which would be suitable for studies of bile acid synthesis.

2. To evaluate the effect of different bile acids on bile acid synthesis as well as key enzymes and nuclear factors involved in bile acid synthesis in cultures of primary human hepatocytes.

3. To study the effect of down- and upregulation of bile acid synthesis in gallstone patients on key enzymes and nuclear factors involved in bile acid synthesis.

4. To screen for unknown polymorphisms and to evaluate the influence of reported polymorphisms in the CYP7A1 gene on enzyme activity, bile acid synthesis and LDL cholesterol levels in serum.

5. To screen for polymorphisms in the CYP8B1 gene that could explain the variability in proportion of CA and CDCA in gallbladder bile.
3 METHODS

3.1 ISOLATION OF RAT HEPATOCYTES

We used male Sprague-Dawley rats weighing about 150 g. The liver was perfused in situ in a non-circulating manner at 37°C, first with 1-200 ml of EGTA buffer at approximately 20 ml/min, secondly with collagenase type XI (Sigma) dissolved in William’s E medium at approximately 10 ml/min. The liver was removed and transferred to a sterile tube containing approximately 30 ml of William’s E medium at 37 °C. The capsule was opened and the cells released into the medium.

3.2 ISOLATION OF HUMAN HEPATOCYTES

Human liver tissue was obtained from three different sources: (I) resection parts from donor livers where the liver was too large for the recipient (mainly from liver transplantation of pediatric patients), (II) patients who were transplanted for familiar amyloidotic polyneuropathy (FAP) where the liver produces a defect prealbumin which causes the disease but otherwise is healthy (III) and patients with liver tumours without underlying parenchymal liver disease undergoing hepatic resection.

To obtain an optimal perfusion of the resected liver the tissue had to have maximum capsule coverage with the smallest cutting surface possible, the ideal part being the lateral segment of the left liver lobe (segments 2 and 3).

The segments suitable by this definition weighed 10-100g. The perfusion was performed by identifying up to three vessels in the cutting area where the perfusion was started with Perfusion fluid I (PF I) containing EGTA buffer in a non-circulating manner for 15 min at
37°C. After this, the liver tissue was perfused with 400 ml Perfusion Fluid II (PF II) containing 200 mg of collagenase type XI (Sigma), in a recirculating manner for approximately 75 min. After the perfusion the liver tissue was transferred to a sterile beaker containing enough cold PF II to cover the specimen. The capsule was cut open near the soft perfused area and the tissue shaken softly in the tissue to release the hepatocytes.

**3.2.1 WASHING AND PLATING OF THE HEPATOCYTES**

After the perfusion step both the human and the rat hepatocytes were treated in the same way. The suspension of cells in medium was strained through a double layer of sterile gauze web and then centrifuged at 50 g for 2 minutes. The cells were then washed twice with William´s E medium. The concentration of cells not trypsine blue stained was counted and a suspension with 7 million cells /ml was prepared. 3.5 million cells were plated onto 60 mm Petri dishes. The dishes had earlier been coated with 200 µL of matrigel (isolated from Engelbreth-Holm-Swarm sarcoma (EHS) [66] and added with 2.5 ml William´s E medium with glutamax and supplemented with insulin (2 IU/L), penicillin G sodium (100 U/ml), Streptomycin sulphate (100ug/ml) and gentamycin (85 ug/ml). The medium was preheated at 37°C.

**3.3 PREPARATION OF MICROSOMES AND ASSAY OF CYP7A1 ACTIVITY**

Microsomes were obtained by differential ultra-centrifugation as described previously [16]. The cells were washed twice with phosphate buffered saline (PBS) and harvested in PBS containing 5 mM EDTA and pelleted by centrifugation (800 g for 2 min). The pellet was homogenized in 0.5 ml of sucrose buffer (50mM Tris-HCl, 0.3 M sucrose and 10 mM EDTA,
pH 7.4) and centrifuged at 20 000 g for 20 minutes at +4°C and the supernatant was centrifuged at 100 000 g for 1 hour at +4°C.

Enzyme activity of CYP7A1 was determined by a method based on isotope dilution-mass spectrometry, using deuterium-labelled 7α-hydroxycholesterol as internal standard and endogenous cholesterol as substrate as described previously[16].

3.4 BILE ACID ANALYSIS

In paper I, bile acids in the cell culture media were analysed as previously described [67, 68]. 2 ml of harvested culture medium, together with deuterium-labelled CA (D₃) and CDCA (D₄) which served as internal standards, 2ml 50% ethanol, 1 mol/L potassium hydroxide were hydrolysed at 125 °C over night. The samples were then diluted with saline solution and extracted twice with etyl ether to remove neutral steroids. After acidification with hydrochloric acid (6 mol/L) to pH 1, the bile acids were extracted with etyl ether. The ether phase was washed with distilled water until pH 7 and then evaporated and methylated with diazomethane. The methylated bile acids were then treated with hexamethyl-silazane and trichlorosilane (TMS) to obtain trimethylsilyl ether derivatives. After this the bile acids were analysed by gas chromatography-mass spectrometry.

In paper II bile acids in the medium were analysed with a simplified version of the method described previously. Briefly, medium (0.5-1.0 mL) was diluted with 2 vol (1.0-2.0 mL) of 0.5 mol/L aqueous triethylamine sulphate, pH 7, followed by extraction on a small column (15x8 mm) of octadecylsilane-bonded silica at 64°C. After washing with water and 10% aqueous methanol, bile acids were eluted with 95% aqueous methanol. Further purification of bile acids was achieved by chromatography on a column (60x4 mm) of the lipophilic anion exchanger triethylaminohydroxypropyl-Sephadex LH-20 (TEAP-LH-20) in bicarbonate form.
After washing with methanol to remove sterols and other neutral lipids, unconjugated bile acids and bile acids conjugated with taurine or glycine were eluted together with 12 mL of 0.3 mol/L acetic acid/ammonium acetate (apparent pH 7) in 95% aqueous methanol. The conjugated bile acids were hydrolyzed by enzymes, re-extracted on a column of octadecylsilane-bonded silica and purified on a column of TEAP-LH-20. After preparing methyl ester/trimethylsilyl ether derivatives, bile acids were analyzed by gas chromatography/mass spectrometry.

3.5 ISOLATION OF TNA IN PAPERS I-III

In Paper I hepatocytes were lysed in a 4 mL of lysis buffer (1% (wt/vol) sodium dodecyl sulphate, 10 mmol/L EDTA in 20 mmol/L Tris-HCL, pH 7.5) and frozen. tNAs were extracted from hepatocytes using proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation, as previously described. The concentration was calculated through density determination with spectrophotometry at 260 nm assuming an RNA/DNA ratio of 2.7:1, then 1 optical density approximates to 43 ng tNAs/µL. In the samples from human hepatocytes, the concentration of tNAs was determined spectrophotometrically, and the DNA concentration was quantified using a specific fluorometric method.

In Paper II and Paper III tNAs were isolated using TRIzol reagent or QuickPrep Total RNA Extraction Kit. RNA concentration was determined by spectrophotometry at 260 nm and the purity and condition of the RNA was determined by spectrophotometry at 280 nm and agarose gel electrophoresis stained with ethidium bromide.
3.6 SOLUTION HYBRIDIZATION OF CYP7A1 mRNA

The relative abundance of specific mRNA for CYP7A1 in Paper I was determined by solution hybridization, using a (\(^{35}\)S) uridine triphosphate(>1000Ci/mmol/L)-labelled complementary RNA probe transcribed \textit{in vitro}. The complementary DNA template for the human probe was an EcoRI-SmaI fragment of the CYP7A1 gene encompassing bases 453 to 740 of the published sequence. The rat samples were assayed with a 184-bp complementary mouse probe. Hybridization, in the presence of 25\% formamide, was carried out overnight at 68°C for the rat samples and at 75°C for the human samples. Standard curves were constructed using synthetic CYP7A1 mRNA.

3.7 RNASE PROTECTION ASSAY

The abundances of CYP27A1 and CYP8B1 mRNAs were quantified using RPA III TM in Paper II. CRNA probes were synthesized using t3- or t7-polymerase and labelled with 35S-UTP. Cyclophilin was used as an internal standard and gave a protected fragment size of 103 bp. The protected fragment size of the probe for CYP27A1 was 297 bp and for CYP8B1 442 bp. The assay was performed according to the protocol provided with the kit. In brief, 10-15 \(\mu\)g of total TNA was precipitated in 2.5 volumes of ethanol and 0.5 NH4Oac with ~80,000 cpm of each probe in -20°C for 15 minutes. Following centrifugation at 15 000 rpm for 15 minutes the pellets were dissolved in 10 \(\mu\)L hybridisation buffer, heated to 95°C for 3 minutes and incubated at 46°C over night. The hybridised RNA samples were then subjected to RNase A and RNase A/T1 diluted 1:50 in Rnase solution at 37°C and inactivated after 30 minutes by adding inactivation/precipitation solution. Following precipitation at \(-20 \, ^\circ\)C for 15 minutes, the samples were centrifuged at 15 000 rpm for 15 minutes and the pellets were air dried and
dissolved in 10 µL of loading buffer. The protected fragments were separated on a denaturating 5% polyacrylamide urea gel and detected and quantified using a Fuji BAS 1800 Phospho-imager.

3.8 REAL TIME PCR

mRNA levels of CYP7A1 and CYP7B1 were determined using singleplex real time PCR. From each group 40 µg RNA was subjected to 5 U of RQ Dnase, 5 µl 10X RQ buffer in a total volume of 50 µl at 37°C for 20 minutes, 20°C for 15 minutes and finally 70°C for 10 minutes. From this 4 µg of Dnased RNA was used for cDNA synthesis together with 2 µl random hexamer primers (100 ng/µl), 1 µl dNTP (10mM of each) in a total volume of 12 µl. After incubation at 65°C for 5 minutes, 25°C for 10 minutes and 42°C for 2 minutes, 2 µl of DTT (0.1M), 1 µl of Rnasin (40 U/µl), 4 µl 5x first strand buffer and 1 µl Superscript II (200 U/µl) were added. The incubation was then continued at 42° C for 50 minutes and at 70° C for 10 minutes. For real time PCR 3 µl undiluted cDNA/sample were used in triplicates.

In Paper II sequences for CYP7A1 primers and probe were kindly provided by Dr Majlis Hermansson, AstraZeneca, Mölndal, Sweden. Reversed prime 5´AGA GCA CAG CCC AGG TAT G 3´, forward primer, 5´TGA TTT GGG GGA TTG CTA TA 3´(300mM) and probe 5´TGG TTC ACC CGT TTG CCT TCT CCT 3´(200 mM). The taqman probe was labelled with FAM and Tamra. For CYP7B1 analysis ABI’s assay-on-demand (Hs00191385_m1) was used. The assays were performed according to ABI user bulletin #2 protocol in a total volume of 25 µl. For endogenous control we used VIC labelled human cyclophilin pre-developed Taqman assay from Applied Biosystems. The assay was performed and analysed using a ABI Prism 7700 sequence detector.
In Paper III analysis for CYP7A1, CYP8B1, CYP27A1, CYP7B1, FXR, SHP, LRH-1(FTF) and HNF-4α were analysed using redeveloped Taqman assays from Applied Biosystems (Hs00167982, Hs00244754, Hs00168003, Hs00191385, Hs00231968, Hs00230853, Hs00222677 and Hs00187067), labelled with FAM and a nonfluorescent quencher. As internal controls human cyclophilin and 18S were used and in the calculations we used cyclophilin which was more stable than 18S.

3.9 DNA PREPARATION

Genomic DNA was prepared from nucleated peripheral white blood cells from frozen whole blood using the Blood & Cell Culture DNA Midi Kit (Qiagen).

3.10 SINGLE-STRANDED CONFORMATION POLYMORPHISM (SSCP)

The SSCP was performed essentially as described before. Briefly, PCR products of approximately 200 bp were denaturated through heating in 100°C boiling water. The fragments were rapidly cooled on ice and 2.5 µl of the PCR product was loaded on a 8.5% polyacrylamide gel containing 5% glycerol and 1xTBE and run in an electrophoretic field of 66 V in a temperature of +4°C for 4 hours. The gels were fixed in 40% methanol/10% acetic acid and silver stained in 12mM AgNO₃. Information regarding primers and assay conditions for nucleotide sequencing and single-stranded-conformation polymorphism (SSCP) analysis of the 6 exons and exon-intron borders of the CYP7A1 gene is available online.
3.11 GENOTYPING WITH RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

Genotyping for the -203C/A and -469T/C polymorphisms was performed using two different restriction fragment length polymorphisms (RFLPs) with non-overlapping primer pairs for PCR amplification, in order to enhance the reliability of the RFLP results. Complete agreement was observed between the two assays for all DNA samples analysed. Information regarding primers and assay conditions of the RFLPs is available as online supplement.

3.12 DNA CONSTRUCTS

Two sets of double stranded oligonucleotides were constructed, constituting the 30 bp sequences around the polymorphic regions of the -203C/A and -469T/C mutations, flanked by BamHI and BglII ends. The double-stranded oligonucleotides were ligated head to tail into a BamHI-digested HCAT vector [69]. Promoter-CAT plasmids were constructed using a 834 bp promoter fragment, spanning from -814 to +20, ligated into a pCAT-Basic vector as described by the supplier (Promega). The promoter fragment was obtained by PCR amplification of a DNA sample from a subject homozygous for the -203C and -469T alleles. Plasmids specific for the -203A and -469C mutations, and a combination of both the -203A and -469C mutations, were generated with the QuickChange site-directed mutagenesis kit (Stratagene Cloning Systems), using the wild-type promoter-CAT plasmid described above.

3.13 ELECTROMOBILITY SHIFT ASSAY (EMSA):

Nuclear extracts were prepared according to Alksnis et al [70]. All buffers were freshly supplemented with leupeptin (0.7 µg/ml), aprotinin (16.6 µg/ml), PMSF (0.2 µM) and 2-mercaptoethanol (0.33 µl/ml). The protein concentration in the extracts was determined by the
method of Kalb and Bernlohr [71]. Incubation for EMSA was conducted as described [72], and the reaction products were applied to 7% (wt/vol) polyacrylamide gel (80:1 acrylamide/N,N’-methylene-bisacrylamide weight ratio), whereafter electrophoresis was performed in 22.5 mM Tris/22.5 mM boric acid/0.5 mM EDTA buffer for 2.5 h at 200 V.

### 3.14 TRANSIENT TRANSFECTION ASSAY

HepG2 cells were cultured in 90-mm dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. Confluent cells were transfected using the calcium-phosphate DNA coprecipitation method. The pSV-β-galactosidase gene (Promega) was cotransfected as an internal control. In all experiments, 5 µg of CAT-construct and 5 µg of β-galactosidase plasmid were added to the medium. CAT activity was analysed using a phosphorimager and β-galactosidase activity was determined as described by the supplier (Promega). CAT levels were expressed in arbitrary units after standardization for β-galactosidase activity. All constructs were tested in duplicates in 4 independent transfection experiments.
4 RESULTS AND CONCLUSIONS

4.1 ESTABLISHING A CELL CULTURE MODEL WITH HUMAN HEPATOCYTES WHICH WOULD BE SUITABLE FOR STUDIES OF BILE ACID SYNTHESIS

In paper I we compared bile acid synthesis in cultures of primary rat and human hepatocytes. The hepatocytes were isolated in a not identical, but very similar way. Fetal calf serum was not added when rat hepatocytes were isolated while a low concentration was used during the isolation, but not during the culture of the human hepatocytes. Perfusion of rat liver was performed in situ but in a sterile beaker using the human specimen. Rat hepatocytes were harvested on the fifth day of culture and human hepatocytes on the eight day. Since the human hepatocytes were isolated and cultured by our collaborator in Australia, enzyme activity assays were not performed on isolated microsomes. The untreated rat hepatocytes showed decreasing bile acid synthesis which progressed during culture time. The synthesis was not restored by supplementary cholesterol. In accordance with a previous study [73] treatment with dexamethasone and thyroid hormone in combination was found to stimulate bile acid synthesis in the cultures of rat hepatocytes. This was paralleled with a raise in CYP7A1 activity and mRNA levels for the enzyme. The increase in bile acid synthesis was mainly due to an increase of non-12 α-hydroxylated bile acids, i.e. CDCA and β-muricholic acid.

In the experiments with human hepatocytes no significant change in bile acid synthesis was seen during hormonal treatment, if anything there was a tendency towards lower synthesis. The human hepatocytes showed a decrease in bile acid synthesis in day 2 which later was restored. The rate of synthesis calculated by weight of liver was similar to the rate found in
in humans. The proportion of CA and CDCA was similar to the proportion found in humans in vivo.

4.1.1 CONCLUSION

Human primary hepatocytes are suitable for studies on human bile acid synthesis. In comparison with rat hepatocytes they appear to better reflect in vivo findings of bile acid synthesis. The optimal time of harvesting and treatment was found to be from day 4 when bile acid synthesis appears to be restored, from the impairment induced by ischemia and perfusion with collagenase.

4.2 EFFECT OF DIFFERENT BILE ACIDS ON BILE ACID SYNTHESIS AND KEY ENZYMES AND NUCLEAR FACTORS INVOLVED IN BILE ACID SYNTHESIS IN CULTURES OF PRIMARY HUMAN HEPATOCYTES

In paper II we used the model of primary human hepatocytes to study the influence of unconjugated and glycine conjugated bile acids on bile acid synthesis and the mRNA levels of CYP7A1, CYP8B1, CYP27A1 and nuclear factor SHP. Bile acids were added day 4 and the cells harvested day 5. Glycine conjugated CDCA, CA and DCA significantly lowered the levels of CYP7A1 mRNA as compared to the controls; also free bile acids lowered the transcript levels. The levels of mRNA for CYP8B1 were significantly lowered by the addition of GCDCA and GDCA but not of GCA. The effect on the levels of mRNA for CYP27A1 was similar to that on CYP8B1. Analysis of the bile acids synthesized was limited by the problem to analyse the synthesis of the specific bile acid that was added, and thus we only studied the effect on the synthesis of the other bile acids. GCDCA was found to be the only bile acid among those tested that had a

35
significant effect on the synthesis of CA in the cultured cells. The addition of GCDCA showed a significantly higher level of UDCA in the cultures. Further experiments, using deuterium-labelled GCDCA, revealed that the UDCA detected originated partly from the added CDCA.

The addition of GCDCA and GDCA both resulted in significantly higher levels of mRNA for SHP.

4.2.1 CONCLUSION

Both glycine conjugated and free bile acids exert negative feedback on bile acid synthesis when added to cultures of primary human hepatocytes. The order of suppressive efficiency was found to be CDCA>DCA>CA>UDCA. The effect on mRNA levels of SHP supports a mechanism by which SHP through activation by FXR (activated by bile acids) is important for the negative feedback of bile acid synthesis performed by bile acids themselves. The rather high increase in mRNA levels of SHP could be due to the fact that the control hepatocytes rather than being in a normal state are daily drained of bile acids by change of media resulting in an upregulated bile acid synthesis.

4.3 EFFECT OF UP AND DOWN REGULATION OF BILE ACID SYNTHESIS IN GALLSTONE PATIENTS ON KEY ENZYMES AND NUCLEAR FACTORS INVOLVED

In paper III we compared the effects of treatment with CDCA and cholestyramine on bile acid synthesis, and its key enzymes, and the nuclear factors involved in the regulation of bile acid synthesis in patients with gallstone disease. The patients were treated with either
cholestyramine (Questran) 8g twice a day or with CDCA 15 mg/kg/day for three weeks before surgery or served as controls.

As expected we saw a significant upregulation of CYP7A1 mRNA levels in the group treated with cholestyramine. There were no significant effects on CYP8B1, CYP27A1 or CYP7B1. A significant increase of mRNA levels for HNF-4α was observed in the group treated with cholestyramine. When we compared the levels of mRNA for CYP7A1 and HNF-4α we found a strong positive correlation between the two measurements. The other nuclear factors (FXR, LRH-1/FTF and SHP) showed no significant changes. This could be due to the fact that nuclear factors are not involved in the regulation of bile acids only but also regulate important cellular events such as proliferation, apoptosis and drug metabolism in the liver. The SHP stimulation observed in the CDCA and DCA treated hepatocytes studied in paper II was not present in our CDCA treated patients. To find out what happened to HNF-4α in the human hepatocytes treated with CDCA we reanalyzed RNA saved from the hepatocyte cultures in paper II. We found that CDCA treatment lowered the levels of HNF-4α mRNA compared to the untreated cells.

4.3.1 CONCLUSION

Upregulation of bile acid synthesis through cholestyramine treatment results in higher levels of mRNA levels for CYP7A1 and HNF-4α with a significant positive correlation between the two mRNA species. The results are consistent with the possibility that HNF-4α is an important stimulatory nuclear receptor involved in bile acid synthesis.
4.4 SCREENING FOR UNKNOWN POLYMORPHISMS AND EVALUATION OF INFLUENCE OF REPORTED POLYMORPHISMS IN THE CYP7A1 GENE ON ENZYMATIC ACTIVITY, BILE ACID SYNTHESIS AND LDL CHOLESTEROL IN SERUM

In Paper IV we evaluated the influence of a previously reported single nucleotide polymorphism in the promoter region of CYP7A1 and also screened for other possible polymorphisms in the exons and intron/exon borders of the gene. The screening of new polymorphisms was performed by SSCP of the promoter region and the exons of the gene including exon/intron borders. In total we analysed 50 patients and subjects including 21 cases in which activity of CYP7A1 had been assayed earlier and 30 in which bile acid turnover had been measured. Twenty subjects were analysed with automatic sequencing of the whole gene including introns.

SSCP revealed no signs of additional polymorphisms in the investigated areas. The automatic sequencing revealed polymorphisms in introns and in the 3′flanking area. These polymorphisms were coupled and always appeared in the same combination, suggesting a highly conserved gene sequence.

We used RFLP analysis to screen for the polymorphism already described by several research groups. We analysed groups of patients and healthy subjects; the groups described above, a group of patients earlier investigated for levels of serum 7α-hydroxy-cholesten-3-one (n=31) and men with previous early coronary heart disease with LDL-cholesterol levels in plasma determined (n=179), healthy men (n=491) and healthy women (n=150) (not reported in the article). The polymorphism did not correlate to any of the above described parameters.

The two polymorphisms were further studied by EMSA and reporter gene construct assays. The only significant finding was that the -469C genotype had higher binding of a nuclear
extract than the -469T and that the transcription rate in the short reporter gene construct was significantly higher in this genotype too. However when analysed together with the coupled genotype this difference disappeared, suggesting that when the two polymorphisms appear together in vivo there is no difference.

4.4.1 CONCLUSION

We could not find any new polymorphism affecting the function of the CYP7A1 gene in subjects with widely different CYP7A1 activity. The polymorphism previously described did not correlate to functional differences in bile acid synthesis in our populations. The possibility of genetical difference between the populations analysed by other groups and those analysed by us, that could influence the importance of the described polymorphism can not be excluded. However, the experimental data suggest that the transcription and activity of CYP7A1 is not influenced by the polymorphisms.

4.5 SCREENING FOR POLYMORPHISMS IN THE CYP8B1 GENE THAT COULD EXPLAIN THE VARIABILITY IN PROPORTION OF CA AND CDCA IN GALLBLADDER BILE

In Paper V we wanted to investigate whether a genetic polymorphism in the CYP8B1 gene could explain the marked differences in CA/CDCA ratios in collected human bile that has been reported.

We screened for polymorphisms in the single exon gene and the nearest part of the promoter region resulting in a 2.4 kb long sequence. We selected 8 patients with a ratio of CA/CDCA ranging from 0.9 to 6.8 assuming that a high ratio indicates a high production of CA and
hence a high activity of CYP8B1. All patients were normolipidemic and had normal thyroid function. We could not find any polymorphisms in the region analysed in any of the patients.

### 4.5.1 CONCLUSION

The marked differences in ratio of CA and CDCA is not explained by a genetic polymorphism in the coding regions or in the nearest promoter region of the CYP8B1.
5 GENERAL DISCUSSION

Bile acid synthesis and bile secretion of free cholesterol represent the major pathways for excretion of cholesterol from the human body and is important to maintain cholesterol balance in the individual. To investigate the mechanisms involved in the regulation of bile acid synthesis, the possibility to both observe the effects of different bile acids \textit{in vitro} in a system of normal human hepatocytes and the \textit{in vivo} situation in humans are complementary to each other. All \textit{in vitro} studies are faced with the challenge of relating the findings to the \textit{in vivo} situation. To really be able to draw firm conclusions from our \textit{in vitro} studies we wanted to establish a cell system containing hepatocytes that are as normal as possible i.e. that they produce bile acids in a pattern similar to the \textit{in vivo} situation, under serum free conditions to eliminate the impact of bile acids or cholesterol added with the serum. If we compare our primary human hepatocyte cultures to the culturing systems of cell lines like Hep G2 cells the bile acids synthesised are predominantly conjugated and the levels of intermediates suggesting incomplete synthesis are low. In cell line cultures the level of unconjugated bile acids and intermediates formed are much higher and not resembling the \textit{in vivo} production of bile acids [61, 62]. This makes the results from cell lines more difficult to interpret. In our studies we culture the hepatocytes on matrigel prepared from EHS-tumours [66]. Analysis of the matrigel has not shown any measurable levels of cholesterol or bile acids. We have not investigated the possible content of growth factors that in theory might influence the hepatocytes. All our cultures were inspected daily to evaluate the loss of cells detached from the matrigel and to detect infections. In the microscope the cells’ appearance resembles the freshly isolated cells i.e. they are round and cover the matrigel surface. When hepatocytes are plated on collagen the cells shape changes and it becomes more fibroblast-like in its appearance. Others have studied the matrigel cultured hepatocytes morphologically more
closely and found that the hepatocytes connect and often after longer time in culture they may even form bile canaliculi. The every day change of medium results in a situation where the hepatocytes are drained of bile acids i.e. resembling the bile fistula situation, with a bile acid synthesis that is upregulated.

When we went on to investigate the effect of both conjugated and unconjugated bile acids we found that CDCA was the most effective suppressor of bile acid synthesis. The capacity to suppress the synthesis was in the following order CDCA>DCA>CA>UDCA. It has been shown that CDCA has the strongest affinity to FXR and this probably explains its potency to reduce the synthesis of bile acids [39]. This order of efficiency mimics the order of hydrophobicity which earlier was thought to be the bile acid property that was important in this aspect. The order of efficacy differs depending on species. In mice, for example, CA is the most potent inhibitor of all bile acids.

We also found that CDCA and DCA induced SHP mRNA which is upregulated by FXR. We could not see any significant effect on FXR mRNA itself. In comparison between treated cells with untreated it should be borne in mind that this is a comparison between hepatocytes with upregulated and downregulated bile acid formation.

In paper III we studied the effect of up- and downregulation of bile acid synthesis in vivo in patients undergoing cholecystectomy. They were treated with either CDCA or cholestyramine three weeks prior to surgery and biliary lipids and mRNA levels of enzymes involved in bile acid synthesis and nuclear factors were analysed in liver biopsies obtained peroperatively. As expected, we found that CYP7A1 was downregulated by CDCA treatment and upregulated by cholestyramine treatment. There were no significant changes in the other enzymes studied. Among the nuclear factors HNF4α mRNA levels were upregulated by cholestyramine treatment and showed significant correlation to the levels of CYP7A1 mRNA levels. The reanalysis of mRNA from primary human hepatocytes in Paper II treated with CDCA showed
lower levels of mRNA for HNF-4α consistent with the findings in Paper III. We could not see any change in SHP, as we did in the *in vitro* study (Paper II). In the *in vivo* study with stimulation of CYP7A1 activity by cholestyramine treatment, the HNF-4α stimulation of the CYP7A1 gene via the SHP-independent pathway appears to dominate over the SHP-dependent pathway. The absence of effects in SHP expression could be due to the fact that SHP is involved in many other cellular regulatory functions such as apoptosis, cell proliferation etc and must be under strict control *in vivo*.

In earlier studies we and others have noticed a marked individual difference in levels of CYP7A1 activity regardless of cholesterol gallstone disease. To address the possibility that these differences are controlled by genetic polymorphisms in the CYP7A1 gene we screened for polymorphisms. Our findings were in accordance with other published results. Wang et al [56] have suggested that these polymorphisms, through altered bile acid synthesis, are responsible for approximately 15% of the differences in LDL-concentrations. However no experimental support for this statement was given. We compared the genotypes in groups of patients and subjects with different markers for bile acid synthesis- CYP7A1 activity, levels of 7α-hydroxy-4-cholesten-3-one, or bile acid turnover- and found no significant differences between the genotypes. Other groups have suggested that these polymorphisms may be responsible for part of the variability in LDL-concentrations in serum. We however found no explanation for the reported differences in terms of altered bile acid synthesis. *In vitro* studies of the polymorphisms with use of a reporter system showed no difference in transcriptional activity when transfected into constructs as a large promoter sequence. No differences between the two forms were seen in binding of nuclear extracts in EMSA. This leads us to believe that the importance of these polymorphisms may be linked to some other regulation of LDL-concentrations in serum or that there may be a population difference between and our subjects and the subjects studied by Wang et al [56] and Couture et al [57]. Hofman et al
have shown that the genetic variation of CYP7A1 correlates with the progression of atherosclerosis and risk of new clinical events [74] and that it also correlates to serum lipid levels in normolipidemic and hypertriglyceridemic individuals [75] and that the genetic variation also affects the HDL-cholesterol levels in serum after cholesterol or cafestol intake [76]. In contrast to Kovar et al [58], however, they could not see any significant changes in LDL-cholesterol levels in serum, after extra cholesterol intake or cholestyramine treatment, when the genotypes were compared [59].

The rate limiting enzyme of the major pathway for bile acid synthesis CYP7A1 is highly conserved and the gene shows high homology through most species investigated. When bile acid synthesis is interrupted or interfered with by homozygous mutations in the major rate-limiting and regulating enzymes involved, the phenotypic changes are often so serious that the individual’s life time, without treatment, is significantly shortened. However, the impact of heterozygous mutations, is probably compensated for by the normal allele.

We and others have noticed marked differences in the ratio of CA and CDCA in gallbladder bile of different subjects, suggesting a difference in CYP8B1 activity. However we could not find any polymorphisms in the gene of the CYP8B1 that explain these differences. A dysfunctional CYP8B1 enzyme is less likely to result in a phenotype with altered bile acid synthesis or LDL-concentrations and may therefore go undetected.

Genetic polymorphisms in the key enzymes may play a certain role in the regulation of bile acid synthesis, but it appears likely that other stimuli, such as negative feedback loops and interference with the nuclear factors involved in the regulation of the expression of the key enzymes in bile acid synthesis dominate, as influential factors. Theoretically, genetic polymorphisms in the genes coding for the nuclear factors involved could also be part of the explanation for the great inter-individual differences observed.
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7 REFERENCES


Yang, Y., et al., On the mechanism of bile acid inhibition of rat sterol 12alpha-hydroxylase gene (CYP8B1) transcription: roles of alpha-fetoprotein transcription...


