Eija Selkälä

ROLE OF α-METHYLACYL-CoA RACEMASE IN LIPID METABOLISM
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ROLE OF α-METHYLACYL-CoA RACEMASE IN LIPID METABOLISM

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University of Oulu, Oulu 2016
Abstract

α-Methylacyl-CoA racemase (Amacr) is an auxiliary enzyme of β-oxidation and participates in the elimination of methyl-branched fatty acids in peroxisomes and in mitochondria and in the synthesis of bile acids in peroxisomes. Amacr catalyzes in reversible manner the isomerization of fatty acyl-CoA esters with a methyl group in the R-configuration to the corresponding S-configuration, which allows them to serve as substrates for the next reaction in their metabolism. The substrates of Amacr include the acyl-CoA esters of 2R-pristanic acid, a metabolite derived from phytol, and 25R-THCA and 25R-DHCA (tri- and dihydroxycholestanolic acid), the bile acid intermediates derived from cholesterol. AMACR-deficiency in humans results in the accumulation of R-isomers of its substrates. Patients with adult onset AMACR-deficiency suffer from neurological disorders. The more severe infantile form of the deficiency is characterized by liver disease. Amacr-deficient mice show a bile acid pattern similar to that of human patients with accumulation of bile acid intermediates in their body. In contrast to humans, Amacr-deficient mice are clinically symptomless on a regular laboratory chow diet. Supplementation of phytol in their diet triggers the disease state with liver abnormalities.

In this study it was shown that in spite of the disruption of a major metabolic pathway, Amacr-deficient mice are able to readjust their cholesterol and bile acid metabolism to a new balanced level allowing them to live a normal life span.

A double knockout mouse model deficient in Amacr and MFE-1 (peroxisomal multifunctional enzyme type 1) was generated in this work. Characterization of this mouse line showed that MFE-1 can contribute to peroxisomal side-chain shortening of C27 bile acid intermediates in both Amacr-dependent and Amacr-independent pathways.

In addition, this work confirmed that Amacr-deficient mice are unable to thrive when phytol is supplemented in their chow. The main cause of death was liver failure accompanied by kidney and brain abnormalities. The detoxification of phytol metabolites in liver is accompanied by activation of multiple pathways and Amacr-deficient mice are not able to respond adequately. The results of this study emphasize the indispensable role of Amacr in detoxification of α-methyl branched fatty acids.

Keywords: bile acids, metabolic regulation, peroxisomal disorders, phytol, α-methylacyl-CoA racemase
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Biokemian ja molekyylilääketieteen tiedekunta; Biocenter Oulu
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä


Tutkimuksen tulokset osoittivat, että Amacr-poistogeennistä hiirien elinkaari ei lyhene huomattavasti yhden tärkeän aineenvaihduntareitin estymisen takia. Tämä on hyvä esimerkkissä siitä, kuinka nisäkkäät pystyvät mukauttamaan aineenvaihduntaansa vastaamaan muuttuneeseen elinympäristöön.

Tässä työssä tuotettiin myös kaksoispoistogeenninen hiirimaallinnun Amacr- ja peroksisomitaalinen monitoimialainen entsyyymi 1- (MFE-1) entsyymit ovat toimimattomat. Tämä hiirillä paljastaa, että MFE-1 pystyy osallistumaan 27:ää hiiltä sisältävien sappihappovälituttoiden sivuaktivoinnin lyhentämiseen sekä Amacr entsyymin kanssa että ilman sitä.


Asiasanat: aineenvaihdunnan säätely, fytoli, peroksisomitaudit, sappihapot, α-metyyliasyyli-koentsyyymi-A-rasemaasi
To my family
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Oulu, March 2016

Eija Selkälä
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ACOX</td>
<td>acyl-CoA oxidase</td>
</tr>
<tr>
<td>ACS</td>
<td>acyl-CoA synthetase</td>
</tr>
<tr>
<td>ALAT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
</tr>
<tr>
<td>Amacr</td>
<td>α-methylacyl-CoA racemase from mammals</td>
</tr>
<tr>
<td>AMN</td>
<td>adrenomyeloneuropathy</td>
</tr>
<tr>
<td>APHOS</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APOA-I</td>
<td>apolipoprotein A1</td>
</tr>
<tr>
<td>ASAT</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ASBT</td>
<td>apical sodium-dependent bile acid transporter</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BA</td>
<td>bile acid</td>
</tr>
<tr>
<td>BAAT</td>
<td>bile acyl-CoA:amino acid N-acyltransferase</td>
</tr>
<tr>
<td>BCOX</td>
<td>branched chain acyl-CoA oxidase</td>
</tr>
<tr>
<td>BSEP</td>
<td>bile salt export protein</td>
</tr>
<tr>
<td>CA</td>
<td>cholic acid</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAR</td>
<td>constitutive androstane receptor</td>
</tr>
<tr>
<td>CDCA</td>
<td>chenodeoxycholic acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>DCA</td>
<td>deoxycholic acid</td>
</tr>
<tr>
<td>DHCA</td>
<td>3α7α-dihydroxycholestanolic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>entgegen</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FXR</td>
<td>farnesoid X receptor</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HMGCR</td>
<td>3-hydroxy-3-methylglutaryl-CoA reductase</td>
</tr>
<tr>
<td>HMGCS1</td>
<td>3-hydroxy-3-methylglutaryl-CoA synthase 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>S</td>
<td>sinister</td>
</tr>
<tr>
<td>SCPx</td>
<td>sterol carrier protein X</td>
</tr>
<tr>
<td>SHP</td>
<td>small heterodimer partner</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>TGR5</td>
<td>membrane-bound G protein-coupled bile acid receptor</td>
</tr>
<tr>
<td>THCA</td>
<td>3α,7α,12α-trihydroxycholestanolic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>TMTD</td>
<td>4R,8R,12-trimethyltridecanoyl-CoA</td>
</tr>
<tr>
<td>TMUD</td>
<td>2R,6R,10-trimethylundecanoyl-CoA</td>
</tr>
<tr>
<td>TYSND1</td>
<td>trypsin domain containing 1</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
<tr>
<td>VLCFA</td>
<td>very long chain fatty acid</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
</tr>
<tr>
<td>X-ALD</td>
<td>X-linked adrenoleukodystrophy</td>
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<tr>
<td>Z</td>
<td>zusammen</td>
</tr>
</tbody>
</table>
List of original articles

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:


Contents

1 Introduction 19

2 Review of the literature 21
   2.1 Peroxisomes ................................................................. 21
      2.1.1 Peroxisomal β-oxidation ............................................ 23
      2.1.2 Origin of pristanic acid .............................................. 27
      2.1.3 β-Oxidation of pristanic acid ...................................... 31
      2.1.4 Disorders of peroxisomal α- and β-oxidation ............... 33
   2.2 Bile acids ......................................................................... 35
      2.2.1 Structure and function .............................................. 35
      2.2.2 Bile acid synthesis .................................................... 36
      2.2.3 Enterohepatic circulation ......................................... 41
      2.2.4 Regulation of bile acid synthesis and enterohepatic
         circulation .............................................................. 42
      2.2.5 Bile acids and regulation of intermediary metabolism ........ 44
   2.3 α-Methylacyl-CoA racemase ............................................. 47
      2.3.1 Structure and reaction mechanism of Amacr ................ 48
      2.3.2 AMACR-deficiency ................................................... 49
      2.3.3 Amacr in cancer ....................................................... 49
      2.3.4 Amacr-deficient mouse model .................................... 50
   2.4 Peroxisomal multifunctional enzymes ................................. 52
      2.4.1 MFE-1 ................................................................. 53
      2.4.2 Mfe-1-deficient mouse model ..................................... 54

3 Outlines of the present study 55

4 Materials and methods 57
   4.1 Animal care (I, II, III) .................................................... 57
   4.2 Mouse models (I, II, III) .................................................. 57
      4.2.1 Generation of Amacr- and Mfe-1-deficient double
         knockout mice .......................................................... 57
   4.3 Analysis of bile acids and fatty acids (I, II, III) ..................... 58
4.4 Lipid analysis (I) ..................................................................................... 59
4.5 Analysis of cholesterol absorption (I) ..................................................... 60
4.6 Analysis of cholesterol synthesis (I) ....................................................... 60
4.7 Plasma and serum analysis (I, II, III) ...................................................... 60
4.8 Histological analysis (II, III) ................................................................. 61
4.9 Molecular biological analysis (I, II, III) .................................................. 62
5 Results 63
5.1 Clinical phenotype of Amacr⁻/⁻ Mfe-1⁻/⁻ double knockout mice (II) ........................................................................................................... 63
5.2 Bile acid analysis (I, II) .......................................................................... 63
5.3 Cholesterol balance studies (I) ......................................................... 64
5.4 Effect of phytol diet on Amacr⁻/⁻ mice (III) ........................................ 67
5.5 Gene expression analysis (I, II, III) ...................................................... 68
5.6 Histological analysis (II, III) ................................................................. 70
5.7 Plasma and serum analysis (I, II, III) ...................................................... 71
6 Discussion 75
6.1 Metabolic adaptation in Amacr⁻/⁻ mice ........................................... 75
6.2 Role of Amacr and MFE-1 in bile acid synthesis in mice .................... 78
6.3 Phytol is lethal for Amacr-deficient mice ............................................. 82
7 Conclusions 87
List of references 89
Original articles 107
1 Introduction

Lipids form a large group of heterogeneous hydrophobic compounds that are essential for life. In living systems, lipids serve as structural molecules of biomembranes, precursors for different hormones, bile acids and second messengers as well as for energy storage. Lipid-bound modifications of proteins are used for their targeting and anchoring to biological membranes. Lipid metabolism is a compartmentalized process that differs between organs, cell types and also within different subcellular organelles. Compartmentalized processes require coordinated interaction between cell organelles governed by the targeted transport of molecules and signal transduction across biomembranes.

A great number of inborn errors and other disorders that affect intermediary metabolism underline the significance of lipid biology in human physiology and pathology. However, the mammalian body possesses complex regulatory networks with high adaptivity to readjust metabolism and allow life to continue. One of the key building blocks of biological lipids are fatty acids. Quantitatively, the major degradative pathway for fatty acyl-CoA esters is $\beta$-oxidation, which is often described as a spiral of four consecutive reactions and in animal cells takes place in both mitochondria and peroxisomes. Methyl-branched lipids and other isoprenoid-derived compounds such as cholesterol must often be metabolized to smaller and more polar metabolites that allow their excretion from the body. Typically $\beta$-oxidation is involved in these processes. However, frequently steric hindrances prevent these compounds from serving as substrates for $\beta$-oxidation enzymes. Therefore, auxiliary enzymes are required, and in these processes $\alpha$-methylacyl-CoA racemase (Amacr) is one of the key enzymes.

Amacr converts the R-isomers of $\alpha$-methyl branched acyl-CoA esters and acyl-CoA esters of bile acid intermediates to S-isomers allowing these substrates to enter the $\beta$-oxidation spiral. A defect in the human gene encoding Amacr results in a disorder with accumulation of 2R-pristanic acid and 25R-bile acid intermediates that causes various neurological symptoms or liver failure. In contrast to human AMACR-deficiency, the previously generated mouse model of Amacr-deficiency is clinically symptomless under standard laboratory conditions (Savolainen et al. 2004). The present study focused on the role of Amacr in mammalian lipid metabolism using the mouse as a model organism. The outcome of this study confirms that the key function of Amacr is the elimination and detoxification of methyl branched fatty acids.
2 Review of the literature

2.1 Peroxisomes

Peroxisomes are intracellular organelles present in all eukaryotic cells except mature erythrocytes. These organelles were first observed in mouse renal cells by Rhodin in 1954 and called microbodies. Christian de Duve introduced the term peroxisome in 1965 after the discovery that these organelles contain oxidases which produce hydrogen peroxide (H$_2$O$_2$) and catalase which degrades H$_2$O$_2$. Peroxisomes were originally considered as cell oddities of unknown function. Active research on peroxisomes since the early 1970s and the recognition of genetic diseases related to human peroxisomes have revealed the crucial role of peroxisomes in human physiology (De Duve & Baudhuin 1966, Vamecq et al. 2014). The most important conserved functions found in peroxisomes of all organisms are the \(\beta\)-oxidation of fatty acids, H$_2$O$_2$ catabolism and inactivation of reactive oxygen species. The enzymes found in mammalian peroxisomes participate in several biosynthetic and catabolic pathways, e.g. in the biosynthesis of cholesterol, bile acids and ether phospholipids, in catabolism of D-amino acids, polyamines and purines, and in the metabolism of glyoxylate. (Smith & Aitchison 2013, Wanders & Waterham 2006).

Peroxisomes are characterized by a proteinaceous matrix surrounded by a single phospholipid bilayer membrane. The dense matrix contains primarily metabolic enzymes. The lipid membrane contains peroxisomal membrane proteins (PMPs), which act primarily as metabolite transporters and peroxins. Peroxins are encoded by PEX genes and are involved in peroxisome biogenesis as well as in membrane and matrix protein import (Fujiki et al. 2014). The diameter of the spherical organelles varies from 0.1 \(\mu\)m to 1.0 \(\mu\)m. Depending on the cell type and the environment, peroxisomes can change their shape, size and number (i.e. peroxisome proliferation) to respond to altering physiological conditions. For instance, physiological stimuli, such as starvation, diabetes mellitus, or high fat diets, induce peroxisome proliferation. Also many chemicals are known to act as peroxisome proliferators. (Smith & Aitchison 2013).

The origin of peroxisomes has been debated for a long time. Currently two pathways of peroxisome biogenesis have been considered: i) de novo synthesis, ii) growth and division (fission). In the de novo synthesis pathway pre-peroxisomal vesicles bud from the endoplasmic reticulum (ER) and subsequently fuse with
each other to form mature peroxisomes. In the growth and fission model a specialized region of the ER containing PMPs forms pre-peroxisomes that fuse with existing mature peroxisomes. After expansion of the peroxisomal membrane, fission occurs. (Smith & Aitchison 2013)

Most PMPs are synthesized on free polysomes in the cytosol and post-translationally imported into the peroxisome membrane. Class I PMPs are directly targeted to peroxisomes through the recognition of a membrane peroxisomal targeting signal (mPTS) in the cytosol by the shuttling receptor Pex19 and through docking of this complex to Pex3 in yeast and Pex19 in mammalian cells at the peroxisomal membrane. Pex19 then mediates the assembly of PMPs into complexes. Class II PMPs can be targeted to peroxisomes by insertion into the ER membrane followed by vesicular transport to peroxisomes. (Smith & Aitchison 2013).

Peroxisomes obtain their matrix proteins by post-translational import as folded proteins from the cytosol by means of peroxisomal targeting signals (PTS), their cognate receptors and import machineries. The majority of the peroxisomal matrix proteins possess a C-terminal targeting sequence PTS1 with a consensus sequence (S/A/C)-(K/H/R)-(L/M) (Elgersma et al. 1996). A small subset of matrix proteins contain near their N-terminus the PTS2 targeting sequence with the consensus sequence (R/K)-(L/V/I)-(X)_{5}-(H/Q)-(L/A) where X is any amino acid (Swinkels et al. 1991). PTS’s are recognized in the cytoplasm by their corresponding soluble import receptors Pex5 (PTS1) and Pex7 (PTS2). The short isoform of Pex5 (Pex5S) interacts with PTS1 proteins only. Pex7 interacts with PTS2 proteins after which cargo-loaded Pex7 forms a complex with the long isoform of Pex5 (5L). The import of matrix proteins is achieved by membrane-associated peroxins, which form the matrix protein import machinery. The PTS1-dependent import mechanism is characterized in more detail. In this model the cargo-receptor complex attaches to peroxisomes through the docking complex on the peroxisomal membrane. Next, PEX5 integrates into the membrane to form a transport channel through which the cargo passes. Finally PEX5 receptors are removed from the membrane and recycled back to the cytosol for another round of import by the exportomer complex. Less is known about the PTS2-dependent import, but it seems to resemble the PTS1-dependent pathway from the docking step onward. (Hasan et al. 2013). A peroxisomal cysteine endopeptidase, Trypsin domain containing 1 (TYSND1), was previously identified to cleave certain PTS1- and PTS2-containing proteins involved in β-oxidation of fatty acids (Kurochkin et al. 2007).
The transfer of metabolites into and out of peroxisomes is accomplished either via specific transporters or membrane channels. Transporters are responsible for the transfer of bulky solutes with molecular masses over 400 Da, e.g., ATP-binding cassette (ABC) transporters of subfamily D (ABCD1, ABCD2 and ABCD3 in mammals) for long chain acyl-CoA derivatives or the ATP transporter for ATP (PMP34). The membrane channels (Pxmp2) allow the nonselective transmembrane passage of small solutes with molecular masses below 400 Da. (Antonenkov et al. 2010, Morita & Imanaka 2012). The essential cofactors CoA, FAD and NAD⁺ that are needed in peroxisomal reactions are synthesized outside the peroxisomes. These cofactors are transported into the peroxisomal matrix by the recently identified transporter SLC25A17 (solute carrier family 25 member 17) (Agrimi et al. 2012).

In humans, defects in genes encoding peroxisomal proteins result in a variety of disorders that can be divided into two groups, including a) the single peroxisomal protein deficiencies (some described in chapter 2.1.2) and b) the peroxisome biogenesis disorders (PBDs), where mutations in different PEX genes result in deficient peroxins and abnormal peroxisome assembly. PBDs are a group of severe developmental brain disorders like Zellweger syndrome spectrum or Rhizomelic chondrodysplasia punctate type 1 that lead to death during childhood. (Fidaleo 2010).

2.1.1 Peroxisomal β-oxidation

Fatty acid degradation in most organisms occurs via the β-oxidation pathway. In animals β-oxidation takes place both in mitochondria and in peroxisomes, whereas in plants and most fungi β-oxidation occurs only in the peroxisomes. The mechanism by which β-oxidation is performed in mitochondria and peroxisomes is similar although the reactions are catalyzed by different enzymes (Eaton et al. 1996). The pathway includes four steps: 1) dehydrogenation, 2) hydration, 3) a second dehydrogenation and 4) thiolytic cleavage. Substrates for mitochondrial β-oxidation are long-, medium- and short chain length saturated and unsaturated fatty acids. In mammals, if the fatty acid chains are too long to be handled by the mitochondria, they are targeted to peroxisomal β-oxidation (Figure 1). This is the case with very long chain (≥C22) fatty acids (VLCFA), 2-methyl branched fatty acids like pristanic acid, and the bile acid intermediates DHCA and THCA. Other substrates for mammalian peroxisomal β-oxidation include polyunsaturated fatty acids, long chain dicarboxylic acids, certain prostaglandins and leukotrienes,
some xenobiotics and metabolites of vitamins E and K. (Wanders & Waterham 2006).

Entering into the \( \beta \)-oxidation cycle requires that the fatty acid be first activated by coupling to CoA. This thioesterification is catalyzed by enzymes belonging to the acyl-CoA synthetase (ACS) family. Only a few of the known ACSs are shown to be peroxisomal. Whether the fatty acids are activated intraperoxisomally or outside the peroxisome and then transported into the organelle has not yet been unequivocally resolved (Watkins & Ellis 2012).

The first step of the \( \beta \)-oxidation cycle in mammalian peroxisomes is oxidation of acyl-CoA to 2-enoyl-CoA catalyzed by acyl-CoA oxidase. In mammals three different acyl-CoA oxidases (ACOX1-3) have been identified. These enzymes are flavoproteins that interact directly with molecular oxygen and generate H\(_2\)O\(_2\). In humans ACOX1 is specific for the CoA esters of straight VLCFAs, whereas ACOX2 preferentially reacts with 2-methyl branched chain acyl-CoAs including pristanoyl-CoA and DHCA/THCA-CoA (Vanhove et al. 1993). ACOX3 is active in rat peroxisomes handling long and very long straight chain and 2-methyl branched chain acyl-CoAs. In humans ACOX3 is not actively expressed (Vanhooren et al. 1997). Because peroxisomal acyl-CoA oxidases are stereospecific acting only on 2S-methylacyl-CoAs, the auxiliary enzyme \( \alpha \)-methylacyl-CoA racemase is needed to convert 2R-methyl fatty acids to the corresponding 2S-fatty acids (Schmitz et al. 1994a, Schmitz et al. 1995).

The next two reactions in peroxisomal \( \beta \)-oxidation are catalyzed by one of two multifunctional enzymes (MFE1-2) that display both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. Although the reactions catalyzed by MFEs are similar, they occur via mirror image stereochemistry. MFE-1 (also known as L-bifunctional protein, LBP, encoded by \textit{EHHADH}) catalyzes the hydration of 2E-enoyl-CoA into 3S-hydroxyacyl-CoA and dehydrogenation of this product to 3-ketoacyl-CoA (Osumi & Hashimoto 1979). MFE-2 (also known as D-bifunctional protein, DBP, encoded by \textit{HSD17B4}) transforms 2E-enoyl-CoA into 3R-hydroxyacyl-CoA and then dehydrogenates this product. Both in humans and in mice MFE-2 has been demonstrated to be the dominant enzyme in the \( \beta \)-oxidation of VLCFAs, pristanic acid, DHCA and THCA (Baes et al. 2000, Ferdinandusse et al. 2006a). The exact physiological role of MFE-1 is currently unclear but it has been shown that it is specifically involved in the peroxisomal \( \beta \)-oxidation of long chain dicarboxylic acids (Houten et al. 2012).
The final step in peroxisomal β-oxidation is the thiolytic cleavage of 3-ketoacyl-CoAs into chain-shortened acyl-CoAs and acetyl-CoA or propionyl-CoA catalyzed by 3-ketoacyl-CoA thiolases. Human peroxisomes contain two different thiolases. 3-Ketoacyl-CoA thiolase (ACAA1) acts on the 3-ketoacyl-CoA esters of straight chain VLCFAs. Sterol carrier protein X (SCPx) thiolase catalyzes the cleavage of 2-methyl branched chain 3-ketoacyl-CoA esters in addition to straight chain substrates (Antonenkov et al. 1997, Seedorf et al. 1994, Wanders et al. 1997).

The β-oxidation of straight chain saturated fatty acids and those acids containing double bonds in the E configuration at even-numbered positions is straightforward. However, the oxidation of fatty acids with double bonds in the Z or E configuration at odd-numbered positions, or double bonds in the Z configuration at even-numbered positions requires auxiliary enzymes. The degradation can be completed by combining the actions of Δ3,Δ5,Δ2,4-dienoyl-CoA isomerase, Δ3,Δ5,Δ2,4-dienoyl-CoA isomerase and 2,4-dienoyl-CoA reductase together with the core enzymes of the β-oxidation pathway (Hiltunen et al. 2003).

The end products of peroxisomal β-oxidation including acetyl-CoA, propionyl-CoA and several medium chain acyl-CoAs including 4,8-dimethylnonanoyl-CoA produced from pristanic acid can be converted to the corresponding carnitine esters via the peroxisomal enzymes carnitine acyltransferase (CRAT) and carnitine octanoyltransferase (CROT) followed by shuttling to mitochondria via the carnitine acylcarnitine carrier for full oxidation (Jakobs & Wanders 1995, Verhoeven et al. 1998). Acyl-CoA esters may also be hydrolyzed within the peroxisome by one of the peroxisomal acyl-CoA thioesters yielding the free acid and CoA (Hunt & Alexson 2002).
Fig. 1. Peroxisomal β-oxidation pathway.
2.1.2 Origin of pristanic acid

Branched chain fatty acids with a methyl group at the carbon three position (β- or 3-methyl branched) are not substrates for β-oxidation. They must first undergo α-oxidation in peroxisomes to remove a single carbon from the carboxyl end and produce fatty acids with the methyl-group at the carbon two position (α- or 2-methyl branched). 2-Methyl branched pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) is derived directly from dietary sources or it is formed via α-oxidation from 3-methyl branched phytanic acid (3,7,11,15-tetramethylhexadecanoic acid). Phytanic acid is solely derived from exogenous sources as phytanic acid itself or as phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol) (Steinberg et al. 1965).

Degradation of phytol

Phytol is a constituent of the chlorophyll molecule in photosynthetic organisms. Humans and mice cannot hydrolyze phytol that is esterified to chlorophyll, but the bacteria present in the gut of herbivores are able to cleave phytol from the porphyrin ring of chlorophyll. The released phytol in the gut of ruminants can further be hydrogenated to dihydrophytol and subsequently oxidized to phytanal and eventually to phytanic acid by the bacterial flora (Figure 2) (Patton & Benson 1966, Van Veldhoven 2010). The excess phytanic acid is stored in neutral and phospholipids in ruminants. Therefore phytanic acid is especially abundant in ruminant fat, meat and dairy products (Lough 1977). Considerable levels of phytanic acid are also present in the animals higher up in the marine food chain where phytol originates from the phytoplankton chlorophyll that is degraded in the gut of zooplankton (Blumer et al. 1963). The absorption and degradation of dietary phytol in non-ruminants is not efficient, which indicates that the major source of phytanic acid in these animals is not from chlorophyll-bound phytol but rather from the phytanic acid that is present in food products (Baxter et al. 1967, Baxter 1968).

The degradation pathway of dietary phytol in mammals (Figure 2) has been discovered only recently, although the localization of the reactions and the identification of the enzymes responsible for the reactions have not been fully identified. The pathway starts with the oxidation of free phytol into phytanal via an unidentified alcohol dehydrogenase. Subsequently, phytanal is converted into phytanic acid by an aldehyde dehydrogenase encoded by the ALDH3A2 gene (van
den Brink et al. 2004). Mutations in the ALDH3A2 gene cause a disease called Sjögren Larsson syndrome (SLS). Clinical symptoms of this disease include ichthyosis, mental retardation and spastic di- or tetraplegia. (Rizzo et al. 1999). Phytanic acid is next converted to its CoA ester and then reduced from phytanoyl-CoA to phytanoyl-CoA (van den Brink et al. 2005) by the peroxisomal enzyme 2E-enoyl-CoA reductase, encoded by the PECR gene (Gloerich et al. 2006). Phytanoyl-CoA is then a substrate for peroxisomal α-oxidation.

Fig. 2. Formation of phytanic acid from phytol.

**α-Oxidation**

In humans α-oxidation (Figure 3) takes place in peroxisomes only. Phytanic acid derived from the diet occurs as a racemic mixture of 3R,7R,11R,15-tetramethylhexadecanoic acid and 3S,7R,11R,15-tetramethylhexadecanoic acid. The estimated daily intake for an adult is 1 mg/kg body weight. Diet derived
phytanic acid is activated to phytanoyl-CoA in an ATP-dependent reaction either in mitochondria, the endoplasmic reticulum or peroxisomes. (Van Veldhoven 2010). The exact mechanism of how phytanic acid is activated is not yet known. Two members of the ACS family are able to perform this action. These are the enzymes ACSL1 (acyl-CoA synthetase long chain family member 1) localized in mitochondria associated membranes, endoplasmic reticulum and cytosol (Lewin et al. 2001), and ACSVL1 (very long chain acyl CoA synthetase, gene SLC27A2) localized at the ER and in the peroxisome (Steinberg et al. 1999). The next step of the α-oxidation pathway occurs in peroxisomes and is catalyzed by phytanoyl-CoA 2-hydroxylase (gene PHYH) using Fe2+, 2-oxoglutarate and O2 as cofactors. This enzyme contains the PTS2, which is cleaved off after import into the peroxisome in a reaction catalyzed by TYSND1 (Kurochkin et al. 2007). The 2-hydroxyphytanoyl-CoA formed is cleaved into pristanal and formyl-CoA by 2-hydroxyacyl-CoA lyase (HACL1). This reaction requires thiamine-pyrophosphate (TPP) and Mg2+ as cofactors. Formyl-CoA is broken down into formate and eventually to CO2 (Foulon et al. 1999). In the last step of the α-oxidation pathway pristanal is transformed into pristanic acid. It is unclear which aldehyde dehydrogenase and which compartment of the cell are responsible for this reaction. One candidate is a splice variant of ALDH3A2 (aldehyde dehydrogenase 3 family member A2) named FALDH-V (fatty aldehyde dehydrogenase), which is specifically targeted to peroxisomes (Ashibe et al. 2007). The stereochemistry of phytanic acid remains unchanged in the course of α-oxidation resulting in both isomers of pristanic acids: 2R,6R,10R,14- and 2S,6R,10R,14-tetramethylpentadecanoic acids.
Similar to other long chain and very long chain fatty acids, phytic acid may also be degraded via microsomal ω-oxidation. In the case of phytic acid, this route becomes important if α-oxidation is impaired as in Refsum disease. The same will happen with other fatty acids when β-oxidation is defective. During ω-oxidation a fatty acid is hydroxylated at its ω-end by members of the cytochrome P450 multienzyme family. Depending on the stereospecificity of the ω-hydroxylase involved, the ω-1-methyl group will be the R or S isoform. Hence, this biochemical pathway might also be Amacr dependent. Subsequently the hydroxyl group formed is converted into an aldehyde by an alcohol dehydrogenase after which it is converted into a carboxyl-group by an aldehyde dehydrogenase. (Komen et al. 2004, Komen et al. 2005).

**ω-Oxidation**
2.1.3 β-Oxidation of pristanic acid

The complete degradation of pristanic acid (Figure 4) requires three cycles of β-oxidation in the peroxisomes and transportation of the intermediate product into mitochondria for further mitochondrial β-oxidation (Verhoeven et al. 1998). Prior to β-oxidation, pristanic acid is activated to pristanoyl-CoA. The enzymes responsible for this reaction have not been identified with certainty. Pristanic acid generated from phytanic acid within peroxisomes could be activated inside the peroxisome by the enzyme ACSVL1, which can activate phytanic acid as well (Jansen et al. 2001, Steinberg et al. 1999). The activation of pristanic acid derived from dietary sources or partly formed outside the peroxisomes is more uncertain, and it remains to be established which ACSL or ACSVL catalyzes this reaction. Recent studies with cultured skin fibroblasts from a patient and with a knockout mouse model confirmed that the transportation of activated pristanoyl-CoA from the cytosol into the peroxisome is mediated via the ABCD3 transporter (Ferdinandusse et al. 2015). Before the β-oxidation of the 2R-isomer of pristanoyl-CoA may proceed inside the peroxisome, it must be racemized by peroxisomal Amacr (Schmitz et al. 1995). The product of the first round of β-oxidation is 4R,8R,12-trimethyltridecanooyl-CoA (TMTD-CoA), which can be further oxidized in the next cycle into 2R,6R,10-trimethylundecanoyl-CoA (TMUD-CoA). With the 2R-configuration, it requires the activity of Amacr to convert it to the 2S-isomer. The third cycle of β-oxidation produces 4R,8-dimethylnonaoyl-CoA. The end product is transported to mitochondria for further oxidation as a carnitine ester (Ferdinandusse et al. 1999) or it is hydrolyzed by one of the peroxisomal acyl-CoA thioesterases and then exported from peroxisomes as a free fatty acid (Ofman et al. 2002). In mitochondria, fatty acids are reactivated to CoA esters and further degraded in mitochondrial β-oxidation. One cycle of mitochondrial β-oxidation results in the production of 2R,6-dimethylheptanoyl-CoA that is once again racemized by Amacr to the S-stereoisomer for completion of β-oxidation (Verhoeven et al. 1998).
Fig. 4. Peroxisomal β-oxidation of pristanic acid.
2.1.4 Disorders of peroxisomal α- and β-oxidation

At present, a genetic disorder in fatty acid α-oxidation is known. Single enzyme deficiencies in peroxisomal fatty acid β-oxidation have been described in six different genetic diseases in humans. α-Oxidation dysfunctions due to disorders in peroxisomal biogenesis or thiamine deficiencies due to various reasons are not discussed here.

Refsum disease

Refsum disease is an autosomal recessive disorder and is the only disorder known to be caused by a deficiency in fatty acid α-oxidation. The disease is caused by a defect in the PHYH enzyme that results in the accumulation of phytanic acid and other substrates for α-oxidation in tissue lipids and in plasma (Jansen et al. 1997). Clinical symptoms include retinitis pigmentosa and night blindness, anosmia, peripheral neuropathy, cerebellar ataxia and nerve deafness (Wierzbicki et al. 2002).

X-linked adrenoleukodystrophy

Adrenoleukodystrophy, which affects the β-oxidation pathway, is the most frequent disorder of peroxisomal fatty acid degradation. This disease can present with different phenotypes depending on the presence and type of neurological findings (Berger & Gartner 2006). The classification of X-ALD is based on the age of onset and affected organs. The most frequent phenotypes are childhood cerebral adrenoleukodystrophy (CCALD) and adrenomyeloneuropathy (AMN). In CCALD, symptoms appear in boys around the first decade with progressive behavioral, cognitive and neurological deterioration leading to a vegetative state. The cerebral phenotype is also seen later in life in adolescence-cerebral ALD (ACALD) or in adult-cerebral ALD. The cerebral phenotype is characterized by inflammatory demyelination in the cerebral white matter, which is absent or mild in AMN. AMN has a much later age of onset and milder progression rate. X-ALD is caused by a defect in the ABCD1 gene. This gene encodes the ALD transporter, which transports CoA-activated VLCFAs from the cytosol into the peroxisome for degradation. Biochemically X-ALD is characterized by the accumulation of VLCFAs in all tissues. The disease affects primarily brain myelin, adrenal cortex and testis. (Berger et al. 2014).
MFE-2-deficiency

MFE-2-deficiency is among the most frequent peroxisomal disorders. Depending on which domain of MFE-2 is affected, the deficiency can be subdivided into three groups: Type 1) total absence of enzyme, type 2) deficiency of the 2-enoyl-CoA hydratase activity, and type 3) deficiency of the dehydrogenase activity. The disease is characterized with multiple abnormalities including craniofacial abnormalities, neuronal migration defects, hypotonia, seizures, and visual and hearing impairment. Non-functional MFE-2 results in the abnormal accumulation of VLCFAs, pristanic acid, as well as bile acid intermediates DHCA and THCA. Type 1 patients suffer from a more severe phenotype and shorter expected life span (<14 months). Type 3 patients can live longer and achieve psychomotor development. (Ferdinandusse et al. 2006a). Recently also very mild presentations of MFE-2-deficiency have been identified in some teenagers (McMillan et al. 2012, Mizumoto et al. 2012, Pierce et al. 2010).

ACOX1-deficiency

ACOX1-deficiency is an autosomal recessive disorder that is characterized by neonatal-onset hypotonia, seizures, failure to thrive, psychomotor retardation, hearing loss, and visual loss with retinopathy. Facial dysmorphism has also been described. Developmental retardation begins at around 2 to 3 years of age, with progressive brain white matter demyelination, psychomotor problems, and peripheral neuropathy resulting in death at a mean age of 5 years. (Poll-The et al. 1988). The straight chain acyl-CoA oxidase enzyme (ACOX1) catalyzes the first step of VLCFA β-oxidation. Biochemically, patients with ACOX1-deficiency show elevated plasma VLCFA levels, whereas they have normal circulating levels of phytanic, pristanic, and di- and trihydroxycholestanolic acids (Ferdinandusse et al. 2007).

AMACR-deficiency

This disorder is described in detail in 2.3.2.
**SCPx-deficiency**

SCPx thiolase activity is required for the breakdown of branched chain fatty acids. SCPx-deficiency has been described only in a single case so far. This 44-year old male patient presented with torticollis, dystonic head tremor, slight cerebellar signs with intention tremor, nystagmus, hyposmia, and azoospermia. Metabolite analyses of plasma revealed an accumulation of pristanic acid. Also abnormal bile alcohol glucuronides were excreted in urine. (Ferdinandusse et al. 2006b).

**ABCD3-deficiency**

The ABCD3 transporter (also known as PMP70) has recently been shown to be involved in the transport of branched chain fatty acids and C27 bile acids into the peroxisome. The first patient with ABCD3-deficiency, a toddler girl, was recently reported. The patient with normal early developmental milestones presented at the age of four with hepatosplenomegaly and severe liver disease and showed accumulation of C27 bile acid intermediates in plasma. (Ferdinandusse et al. 2015).

### 2.2 Bile acids

#### 2.2.1 Structure and function

Bile acids are derived from the catabolism of cholesterol in liver (Figure 5). Bile acids are composed of fused carbon rings consisting of three 6-carbon rings, one 5-carbon ring, and one 5-carbon side chain. Bile acids have hydroxyl groups and the carboxyl group facing one side of the carbon lattice to form a hydrophilic face, which opposes the other hydrophobic face. This amphipathic structure gives bile acids detergent properties. In mammals, most bile acids are C24-5β-bile acids, which are conjugated to the amino acids glycine or taurine to form bile salts with increased solubility (Li & Chiang 2014).

Primary bile acids are synthesized from cholesterol in the liver and may be converted to secondary bile acids by enzyme activities in intestinal bacteria. The human bile acid pool consists of the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA), the secondary bile acid deoxycholic acid (DCA) and a trace amount of lithocholic acid (LCA). The bile acid pool in mice consists mostly of primary CA and α- and β-muricholic acid (α-,β-MCA) and secondary...
ω-MCA. The bile acid pool in humans is more hydrophobic whereas in mice it is hydrophilic, which has a significant effect on the activation of bile acid-responsive receptors. (Chiang 2009).

Bile acids play multiple roles in the physiology of mammals. Together with cholesterol and phospholipids, bile acids form mixed micelles in the small intestine that facilitate solubilization, digestion and absorption of lipids and lipid-soluble vitamins (A, D, E and K). Bile acids as detergent molecules are also required for the solubilization of cholesterol in bile, thus preventing cholesterol crystallization and gallstone formation. Bile acids stimulate bile flow from hepatocytes into the bile canaliculi and gall bladder, and have antibacterial properties that maintain sterility in the biliary tract. (Inagaki et al. 2006). Biliary secretion of the bile acids facilitates hepatobiliary secretion of endogenous and xenobiotic metabolites. Bile acid synthesis is the major route for excretion of excess cholesterol and is important in whole-body sterol homeostasis. In adult humans each day approximately 0.5 g of cholesterol is converted into bile acids. Recent findings show that bile acids are also important signaling molecules that regulate multiple metabolic pathways and their own synthesis. Abnormal bile acid synthesis and metabolism is associated with cholestasis, gallstones, inflammation, malabsorption of lipids and lipid-soluble vitamins, atherosclerosis, neurological diseases and various inborn errors. (de Aguiar Vallim et al. 2013).

2.2.2 Bile acid synthesis

Bile acids are synthesized from cholesterol through two major pathways, the classic and the alternative pathway in different compartments of the cell (Figure 5). Microsomal and cytosolic enzymes are involved in the modification of the cholesterol ring structure. Oxidation of the sterol side chain occurs in the mitochondria, and finally, both the cleavage of the side chain and subsequent conjugation with glycine or taurine to primary bile acids take place in peroxisomes (Russell 2003). The classic (also called neutral) pathway accounts for about 90% in humans and 75% in mice of the total bile acid synthesis. The products of this pathway are CA and CDCA. The alternative (also called acidic) pathway generates CDCA via oxysterol intermediates. Oxysterols are formed both in hepatic and extrahepatic tissues where the side chain of the cholesterol is hydroxylated at three different positions. The alternative pathway accounts for about 10% in humans and 25% in mice of the total bile acid synthesis. (de Aguiar Vallim et al. 2013).
Initiation

Cholesterol 7α-hydroxylase (CYP7A1, in the ER), a cytochrome P450 enzyme localized exclusively in the liver, is the rate-limiting enzyme that begins the classic pathway by converting cholesterol to 7α-hydroxycholesterol. Sterol 27-hydroxylase (CYP27A1, in mitochondria) which is expressed in the liver, macrophages and most other tissues initiates the major alternative pathway. This enzyme produces 27-hydroxycholesterol but it can also hydroxylate cholesterol at carbons 24 and 25 to form 24-hydroxycholesterol and 25-hydroxycholesterol (Lund et al. 1993). Other minor pathways are initiated by cholesterol 25-hydroxylase (CH25H, in the ER) to produce 25-hydroxycholesterol in the liver and 24-hydroxylase (CYP46A1, in the ER) to produce 24S-hydroxycholesterol in the brain and in the liver. 24-hydroxylation contributes little to overall bile acid synthesis but it is an important pathway because it contributes to cholesterol secretion from brain (Lund et al. 2003). The 27-hydroxycholesterol and 25-hydroxycholesterol are then further hydroxylated by oxysterol 7α-hydroxylase (CYP7B1, in the ER). The hydroxylation of 24-hydroxycholesterol is catalyzed by another oxysterol 7α-hydroxylase (CYP39A1, in the ER). (Russell 2003). Unlike CYP7A1, oxysterol 7α-hydroxylases are constitutively expressed and not sensitive to bile acid or cholesterol status (Li-Hawkins et al. 2000, Schwarz et al. 2001).

Ring structure modification

The 7α-hydroxylated intermediates of both pathways are handled by 3β-hydroxy-Δ5-C27-sterol oxidoreductase (HSD3B7, in the ER). The ring structure modification of the products of HSD3B7 is then continued. Depending on the action of 12α-hydroxylase (CYP8B1, in the ER), the intermediates are eventually converted to CA. Alternatively, without the action of CYP8B1, bile acid intermediates are eventually converted to CDCA in humans or MCA in mice. The products of HSD3B7, whether or not they are acted upon by CYP8B1, are next subjected to reduction of the double bond in a ring structure by the cytosolic enzyme Δ4-3-ketosteroid 5β-reductase (AKR1D1). In the final step of the ring structure modification the enzyme 3α-hydroxysteroid dehydrogenase (AKR1C4) catalyzes the reduction of the 3-keto-group to an alcohol-group. (Russell 2003).
**Oxidation and shortening of the side chain**

The products of ring structure modification are further subjected to oxidation and shortening of the sterol side chain. The same mitochondrial enzyme CYP27A1 that initiates bile acid synthesis through formation of 27-hydroxycholesterol introduces a hydroxyl-group at carbon 27 and then oxidizes this group to an aldehyde and converts the sterol intermediate eventually to DHCA or THCA. These C27 bile acid intermediates have to be activated to their CoA-thioester and transported into peroxisomes before shortening of the side chain. Two enzymes from the ACS family have been identified that can perform this activation. The first is liver specific bile acyl-CoA synthase (BACS, encoded by \textit{SLC27A5}), which is located exclusively at the ER. It accepts both C27- and C24-bile acids as substrates. The second is very long chain acyl-CoA synthase (VLCS, encoded by \textit{SLC27A2}) expressed primarily in liver and kidney and localized at the ER and in the peroxisome. This enzyme cannot handle primary C24-bile acids, but is able to activate C24:0, C16:0, phytanic and pristanic acids. (Mihalik \textit{et al.} 2002, Steinberg \textit{et al.} 1999). It is not clear which of the two synthases is actually activating C27 bile acid intermediates. ABCD3 has been suggested to be the transporter of the DHCA- and THCA-CoAs into the peroxisome (Ferdinandusse \textit{et al.} 2015).

**Peroxisomal steps in bile acid synthesis**

**Conjugation**

Prior to their secretion into the bile, primary bile acids are conjugated at their side chains with glycine or taurine, which further enhances their hydrophilicity. The amidation of the CA-CoA and CDCA-CoA to glycine or taurine is catalyzed by the peroxisomal bile acyl-CoA: amino acid N-acyltransferase (BAAT) (Pellicoro et al. 2007). In mice C24-bile acids are conjugated with taurine and in human with both taurine and glycine (Falany et al. 1994, Falany et al. 1997). C24 bile acids are efficiently conjugated, the ratio of glycine to taurine being 3:1, but C27 bile acid intermediates are poor substrates for BAAT (Ferdinandusse et al. 2009b). The taurine- and glycine-esters are transported out of the peroxisome into the cytosol by as yet an unknown mechanism (Kemp et al. 2011). At physiological pH, conjugated bile acids are fully ionized and bile acids exist as anionic salts.
Fig. 5. Enzymes of bile acid synthesis.
2.2.3 Enterohepatic circulation

Newly synthesized bile acids are secreted from hepatocytes into bile canaliculi and stored in the gallbladder. Unconjugated bile acids can diffuse from hepatocytes across the apical membrane, but conjugated bile acids require a canalicular bile salt export protein (BSEP; ABCB11) for transportation. The other compounds of bile, namely phospholipids and cholesterol are also secreted from hepatocytes into bile canaliculi via specific transporters ABCB4 and ABCG5/ABCG8, respectively. (Halilbasic et al. 2013). In response to feeding, bile enters the duodenum, which contributes to the emulsification of dietary lipids. In the distal ileum bile acids are efficiently absorbed (95% absorbed) either by passive diffusion or by the active transporter apical sodium-dependent bile acid transporter (ASBT), which is present on the enterocyte brush border. Some of the secreted bile acids that escape resorption are dehydroxylated at the 7α position by gut bacterial flora to produce secondary bile acids (Ridlon et al. 2006). The intestinal bile acid-binding protein (IBABP) facilitates the movement of bile acids across the enterocyte to the basolateral membrane where they are exported into the portal blood via by the organic solute transporter α and β heterodimer (OSTα/OSTβ). Recent evidence suggests that OSTα/OSTβ also plays a role in reuptake of bile acids in the kidney (Soroka et al. 2010). Reabsorbed bile acids are transported back to the liver via the portal blood circulation, and taken up at the sinusoidal membrane of the hepatocytes by sodium taurocholate cotransporting polypeptide (NTCP) and organic anion transporters (OATPs). In the hepatocytes, bile acids are re-conjugated and then re-secreted together with newly synthesized bile acids to complete one cycle of the enterohepatic circulation (Figure 6). (Alrefai & Gill 2007, Dawson et al. 2009, Hagenbuch & Stieger 2013). The exception is the LCA that is usually present at low levels and is hepatotoxic at elevated concentrations. The LCA that is returned to the liver is sulfated prior to secretion into bile and excretion in the feces (Hofmann 2004). Recycling of bile acids in the enterohepatic circulation may occur 4–12 times per day. Bile acids lost in the feces are replaced by de novo synthesis. On average, the human liver synthesizes 0.2–0.6 g of bile acids daily.
2.2.4 Regulation of bile acid synthesis and enterohepatic circulation

Farnesoid X receptor FXR

Bile acid synthesis and the enterohepatic circulation of bile acids are tightly regulated in order to maintain a constant bile acid pool size and hence this serves as an important physiological route for regulation of whole-body lipid metabolism. The maintenance of bile acid homeostasis is essentially performed through complex transcriptional networks involving nuclear receptors (Table 1) and other signaling pathways (Chiang 2009).

Bile acids are regulators of their own synthesis as seen in the early studies where oral administration of bile acids to rodents was shown to reduce CYP7A1
enzyme activity and bile acid synthesis (Shefer et al. 1968). The role of the farnesoid X receptor (FXR), in maintaining control of bile acid levels was discovered in studies with Fxr<sup>-/-</sup> mice (Sinal et al. 2000). FXR is a member of the nuclear receptor superfamily and is highly expressed in the enterohepatic tissues. After translocation to the cell nucleus, heterodimerization to the retinoid X receptor (RXR) and binding to the hormone response element on DNA, FXR mediates the negative feedback loop from bile acids to downregulate the expression of enzymes involved in bile acid synthesis. FXR activation induces the expression of the small heterodimer partner (SHP), which in turn inhibits transactivation of CYP7A1 and CYP8B1 by the transcription factors hepatocyte nuclear factor 4α (HNF4α) and liver-related homolog 1 (LRH1) at the bile response element. (Li & Chiang 2014).

An additional feedback repression of bile acid synthesis involves FXR regulation of fibroblast growth factor 19 (FGF19) in humans and FGF15 in mice. Bile acid activation of FXR results in secretion of FGF19/15 from hepatocytes and enterocytes. FGF19/15 then activates the hepatic FGF receptor-4 and membrane-bound co-receptor protein complex (FGFR4/β-Klotho complex) on hepatocytes leading to suppression of CYP7A1 via a c-Jun N-terminal kinase dependent mechanism. (de Aguiar Vallim et al. 2013).

In the liver, activated FXR induces the expression of BSEP, ABCB4 and ABCG5/ABCG8, which enhance the transport of bile acids, phospholipids and cholesterol, respectively, into the biliary canaliculi. At the same time, the transport of bile acids from the portal blood into the hepatocytes is limited by FXR which represses the expression of bile acid importers NTCP and OATP in a SHP dependent manner. Within the intestine, bile acid activated FXR reduces bile acid absorption via down-regulation of the ASBT, promotes bile acid movement across the enterocyte via IBABP and promotes recycling of bile acids to the liver via OSTα/OSTβ. (Calkin & Tontonoz 2012). The potency of certain bile acids to activate FXR depends on the hydrophobicity of the bile acid. The hydrophobic bile acid CDCA is the most efficacious endogenous FXR ligand, followed by LCA, DCA and CA, whereas the hydrophilic bile acids such as ursodeoxycholic acid (UDCA) and MCA do not activate FXR (Parks et al. 1999).

A novel study identified a new transcription factor, V-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog G (MafG), which is a direct target gene of FXR. The FXR-MafG pathway controls the transcription of multiple genes involved in both the classic and alternative pathways of bile acid
synthesis (i.a. Cyp8b1, Cyp7b1, Acox2, Akr1d1, Akr1c14, Scp2) and bile acid transport (Ntcp) (de Aguiar Vallim et al. 2015).

**Pregnane X receptor (PXR), vitamin D receptor (VDR), constitutive androstane receptor (CAR)**

Other nuclear receptors, such as the specific bile acid activated pregnane X receptor (PXR) and vitamin D receptor (VDR) can also regulate bile acid synthesis by suppressing CYP7A1. PXR is especially activated by the hepatotoxic bile acid LCA (Staudinger et al. 2001). The constitutive androstane receptor (CAR) can also regulate target genes in bile acid metabolism. There is no evidence that bile acids can activate CAR, but CAR agonists have been shown to repress CYP7A1. Like PXR, CAR may be involved in detoxification of xenobiotics and toxic LCA. Mice studies have shown that the VDR acts as a bile acid sensor in the intestine and represses CYP7A1 via FGF15. VDR also protects the intestine from bile acid toxicity by stimulating the sulfation of potentially toxic bile acids. (Li & Chiang 2014).

### 2.2.5 Bile acids and regulation of intermediary metabolism

**Membrane-bound G protein-coupled bile acid receptor (TGR5)**

Another important bile acid dependent signaling mechanism that dominates with FXR in the regulation of glucose, lipid, and energy metabolism was found about a decade ago (Kawamata et al. 2003, Maruyama et al. 2002). This is a membrane-bound G protein-coupled bile acid receptor (TGR5). TGR5 is expressed in many organs and tissues with high expression in hepatic Kupffer cells and cholangiocytes as well as immune cells, and gallbladder epithelial cells. The other tissues where TGR5 is also expressed include brown adipose tissue, intestinal enteroendocrine cells that secrete the incretin hormone glucagon-like peptide-1 (GLP-1), enteric neurons and the central nervous system. (Duboc et al. 2014). Activation of TGR5 by bile acids results in increased intracellular levels of cAMP and activation of protein kinase A, leading to increased phosphorylation of target proteins (de Aguiar Vallim et al. 2013). Results of TGR5 activation are cell type specific. In macrophages TGR5 activation suppresses the release of proinflammatory cytokines (Pols et al. 2011). TGR5 present on the biliary tree
controls fluid and chloride secretion by the epithelial cells and the postprandial relaxation of the gallbladder in response to cholecystokinin (CCK) (Keitel & Haussinger 2012). Recent studies with Tgr5−/− mice have shown that normal peristalsis and transit time in the colon are dependent of the activation of TGR5 by intestinal bile acids on enterochromaffin cells and inhibitory colonic neurons (Alemi et al. 2013). TGR5’s role in energy metabolism was seen in mouse experiments where supplementation of CA to a high-fat diet resulted in elevated blood levels of bile acids that attenuated diet induced obesity and insulin resistance. The mechanism was postulated to involve bile acid-dependent activation of TGR5 in brown adipose tissue, which, via increased cAMP, activated the type 2 iodothyronine deiodinase leading to increased levels of active thyroid hormone (T4) and induction of genes involved in energy metabolism. (Watanabe et al. 2006). The bile acid mediated activation of TGR5 on intestinal enteroendocrine L cells stimulates GLP-1 production into the circulation. GLP-1 is known to promote insulin secretion and thus regulate glucose homeostasis. (Katsuma et al. 2005).

Interruption of the enterohepatic circulation of bile acids has beneficial effects for patients with hyperlipidemia and type 2 diabetes. This is achieved by the administration of bile acid sequestrants, such as cholestyramine, colestipol and colesevelam, which have long been used for the treatment of hypercholesterolaemia. These resins bind bile acids in the intestinal lumen preventing their reabsorption in the ileum and promoting bile acid excretion in the feces. Increased levels of bile acids in the colon activate colonic TGR5, enhance GLP-1 levels and lead to increased insulin sensitivity and decreased glucose levels in blood. (Harach et al. 2012). Metabolic improvements may also be seen in patients after ileal bypass surgery, used as a therapeutic option for morbid obesity. It has been found that after weight-loss surgery, the total bile acid pool increases and elevates TGR5 signaling, which contributes to improved metabolic control (Kohli et al. 2013, Patti et al. 2009).

Liver X receptor (LXR)

The acidic pathway of bile acid synthesis is an important pathway generating regulatory oxysterols, oxidized derivatives of cholesterol. Oxysterols, including 22R-hydroxycholesterol, 24S-hydroxycholesterol, 24S,25-epoxycholesterol, 20S-hydroxycholesterol, and 27-hydroxycholesterol, are natural ligands for nuclear receptor liver X receptors α and β (LXRα, LXRβ). LXRα is expressed in tissues
with high metabolic activity, such as liver, adipose tissue and macrophages, whereas LXR\(\beta\) is ubiquitously expressed. LXRs function as ligand activated sterol sensors, and when cellular oxysterols accumulate as a result of increasing concentrations of cholesterol, LXRs enhance the transcription of genes involved in the regulation of cholesterol metabolism and protect cells from cholesterol overload. Upon activation by ligands, LXRs bind to the response element of the target gene as heterodimers with RXR. (Calkin & Tontonoz 2012). In mice LXR\(\alpha\) induces cholesterol catabolism by binding to the promoter of CYP7A1 thus upregulating gene expression (Peet et al. 1998). The human CYP7A1 promoter does not bind LXR\(\alpha\). In humans LXR\(\alpha\) agonist treatment suppresses the expression of CYP7A1 in primary human hepatocytes. The repression may be in part due to the direct induction of SHP that has a repressive effect on CYP7A1 via LRH1. (Goodwin et al. 2000, Goodwin et al. 2003).

In peripheral cells such as tissue macrophages, LXR activation induces the expression of proteins involved in reverse cholesterol transport, which is a pathway where accumulated cholesterol is transported from peripheral tissues to the liver for biliary secretion. These proteins include cholesterol efflux transporters ABCA1 and ABCG1, apolipoprotein E (APOE) and a lipoprotein remodeling enzyme phospholipid transfer protein (PLTP). Recent studies demonstrated that LXR negatively regulates the LDL receptor (LDLR) mediated cholesterol uptake pathway. The target gene in this pathway is the inducible degrader of LDLR (IDOL). IDOL is an E3 ubiquitin ligase that marks LDLR for degradation and attenuates LDL uptake by cells (Zelcer et al. 2009). As a side note, another major post-translational regulator of LDLR is proprotein convertase subtilisin kexin type 9 (PCSK9), which is transcriptionally regulated by HNF1\(\alpha\) in cooperation with the sterol regulatory element-binding protein-2 (SREBP-2) and has become a target of new antibody based cholesterol lowering drugs (Lagace 2014). In the intestine, LXR inhibits cholesterol absorption by inducing the expression of ABCG5 and ABCG8. LXR may have an indirect role in attenuation of the expression of Niemann-Pick C1-like-1 (NPC1L1), a protein that is important for intestinal cholesterol absorption, but the mechanism behind that is not clarified yet. (Calkin & Tontonoz 2012).
Table 1. Nuclear receptors in bile acid metabolism.

<table>
<thead>
<tr>
<th>Nuclear receptor</th>
<th>Ligand</th>
<th>Major target genes</th>
<th>Regulation</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXR</td>
<td>Bile acids</td>
<td>CYP7A1</td>
<td>↓</td>
<td>Bile acid synthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BSEP</td>
<td>↑</td>
<td>Bile acid transport into the gall bladder</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NTCP</td>
<td>↓</td>
<td>Bile acid uptake into the hepatocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASBT</td>
<td>↓</td>
<td>Intestinal bile acid absorption</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IBABP</td>
<td>↑</td>
<td>Intracellular bile acid transport</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OSta/OSTβ</td>
<td>↑</td>
<td>Bile acid secretion into the portal blood</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FGF15/FGF19</td>
<td>↑</td>
<td>Bile acid synthesis inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SHP</td>
<td>↑</td>
<td>Bile acid synthesis inhibition, bile acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>secretion into the portal blood inhibition</td>
</tr>
<tr>
<td>LXR</td>
<td>Oxysterols</td>
<td>Cyp7a1</td>
<td>↓</td>
<td>Bile acid synthesis (only in mice)</td>
</tr>
<tr>
<td>PXR</td>
<td>Bile acids</td>
<td>CYP7A1</td>
<td>↓</td>
<td>Bile acid synthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FGF15/FGF19</td>
<td>↑</td>
<td>Bile acid synthesis inhibition</td>
</tr>
<tr>
<td>VDR</td>
<td>Bile acids</td>
<td>CYP7A1</td>
<td>↓</td>
<td>Bile acid synthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FGF15</td>
<td>↑</td>
<td>Bile acid synthesis inhibition</td>
</tr>
</tbody>
</table>

For citations see main text.

2.3 α-Methylacyl-CoA racemase

α-Methylacyl-CoA racemase catalyzes the chiral inversion of CoA activated 2R- and 2S-methyl fatty acid substrates with diverse structures including pristanoyl-CoA and its chain-shortened derivatives, and 25R-stereoisomers of bile acid intermediates like DHCA-CoA and THCA-CoA. The importance of Amacr arises because only stereoisomers with the 2-methyl group in the S-configuration can be degraded in peroxisomal β-oxidation (Schmitz et al. 1994a, Schmitz et al. 1995). The absence of Amacr activity due to mutations in the human AMACR gene results in a neurological disorder caused by the accumulation of 2R-methyl branched chain substrates, which indicates that Amacr activity is essential for the metabolism of these substrates (Ferdinandusse et al. 2000a). Amacr is shown to be identical to the 2-arylpropionyl-CoA epimerase that converts 2-arylpropionyl-CoA esters of the non-steroidal anti-inflammatory drug family (e.g. ibuprofen) from the R-enantiomer to the S-enantiomer. The chiral conversion of these drugs is required for full pharmacological activity, since only the S-enantiomer is active as a cyclo-oxygenase (COX) inhibitor (Brugger et al. 1996, Reichel et al. 1995).

The human AMACR gene is located on the short (p) arm of chromosome 5 at position 13 (5p13.2-q11.1), and in mouse Amacr is located on chromosome 15, in region 15B1. AMACR has several different splice variants that have been found in
human normal and tumorous prostate tissues. Variants include the inclusion or exclusion of exon 3, the use of an alternative exon 5, and alternative splicing within the alternative exon 5. The most abundant variant (also produced at the highest levels in prostate cancer) is the full length \textit{AMACR IA} that contains 5 exons and codes for a protein of 382 amino acids (Mₚ 42 kDa). The physiological significance of the other splice variants is presently unknown (Mubiru \textit{et al.} 2004, Ouyang \textit{et al.} 2011).

In humans, mice and rats, Amacr is localized both in peroxisomes and mitochondria (Ferdinandusse \textit{et al.} 2000b, Kotti \textit{et al.} 2000, Schmitz \textit{et al.} 1995). The human AMACR IA was shown to contain a polypeptide KASL at the C-terminal end that functions as PTS1. The PTS1 sequence is absent from other splice variants but all variants possess a common N-terminal mitochondrial targeting sequence (Amery \textit{et al.} 2000).

Based on EST (expressed sequence tags) database analysis AMACR is expressed in most human tissues. The Amacr protein has been detected in liver, kidneys and to a lesser extent in the intestinal tract and salivary glands by Western blot analysis (Schmitz \textit{et al.} 1995, Zha \textit{et al.} 2003). In rats the highest activity is seen in the liver, Harderian gland, kidneys and intestinal mucosa (Schmitz \textit{et al.} 1997, Van Veldhoven \textit{et al.} 1997).

\textbf{2.3.1 Structure and reaction mechanism of Amacr}

Sequence comparison studies have shown that Amacr belongs to the family III CoA-transferases also referred to as the CaiB-BaiF family (Heider 2001). The enzyme is highly conserved sharing 81% and 77% sequence identity with the rat and mouse orthologs, respectively (Ferdinandusse \textit{et al.} 2000a, Schmitz \textit{et al.} 1997). Amacr from \textit{Mycobacterium tuberculosis} (MCR) has approximately 40% sequence identity to human and rat Amacr (Savolainen \textit{et al.} 2005).

No structure of mammalian Amacr has been reported but several crystal structures of MCR-substrate complexes have been obtained. MCR consists of α/β-secondary structures and two peptide chains are interlinked characteristic of type III CoA transferases. The active site of MCR is at the dimer interface where His126 and Asp156 act as catalytic amino acids. This hydrophobic surface is conserved in the homologous mammalian racemases (Lee \textit{et al.} 2006, Savolainen \textit{et al.} 2005, Sharma \textit{et al.} 2012).

Based on structural studies, a reaction mechanism has been proposed for MCR. A racemase catalyzed reaction requires no cofactor or co-substrates. The
catalytic cycle is characterized by a planar enolate intermediate having a negative charge on the thioester oxygen. The inversion of the chirality of the Cα-carbon of an enantiomeric 2-methyl branched fatty acyl-CoA is achieved by a 1,1-proton transfer mechanism exchanging the position of the Cα-proton and acyl moiety by a catalytic acid/base-pair. Curiously, Amacr catalyses also the exchange of straight chain acyl-CoA α-protons (Sattar et al. 2010). The MCR has separate binding pockets for S- and R-acyl chains and during the catalysis the acyl group moves over a hydrophobic methionine rich surface where the 2-methyl moiety and the CoA group remain fixed. (Bhaumik et al. 2007, Sharma et al. 2012).

2.3.2 AMACR-deficiency

AMACR-deficiency is a rare peroxisomal single enzyme deficiency inherited in an autosomal recessive pattern. The prevalence of this disorder is unknown. To date only 10 adult and 4 child patients have been described. The deficiency of Amacr results in the accumulation of R-isomers of pristanic and phytanic acids and DHCA and THCA bile acid intermediates. The clinical phenotype is diverse and can be divided into two age groups. Early onset disease resembles bile acid deficiency with neonatal cholestasis and giant cell hepatitis (Setchell et al. 2003). Coagulopathy and vitamin-K deficiency have also been described (Van Veldhoven et al. 2001). In late onset disease neurological symptoms predominate including encephalopathy, sensory and motor neuropathy, seizures, cerebellar ataxia, and retinitis pigmentosa. In two male patients the presence of hypogonadism has been described. Symptoms are suggested to exist mainly due to the accumulation of toxic pristanic acid. The predominant mutation in Amacr-deficient patients is a missense mutation in the AMACR gene: c.154 T>C [p.Ser52Pro]. Other mutations described are c.559 G>A [p.Gly187Arg], c.367 G>A [Asp123Asn] and c.320 T>C [p.Leu107Pro] (Ferdinandusse et al. 2000a, Haugarvoll et al. 2013).

2.3.3 Amacr in cancer

Amacr is greatly overexpressed in all prostate cancers and in a number of other cancers such as renal, breast, and colon cancers (Jiang et al. 2003a, Jiang et al. 2003b, Zhou et al. 2002). The high levels of overproduction in prostate cancer indicate that antibody-based methods could be used to diagnose prostate cancer from biopsy samples with the marker known as P504S (Jiang et al. 2001).
Epidemiological evidence and some laboratory studies show that dietary intake of phytanic acid correlates with prostate cancer risk (Wright et al. 2011, Wright et al. 2012), and dietary branched chain fatty acids increase the production of Amacr enzyme and its catalytic activity in prostate cancer cells (Kumar-Sinha et al. 2004, Mobley et al. 2003). However, one recent study investigating the relationships among dietary intake, serum and tissue concentrations of phytanic acid and Amacr expression in the benign human prostate challenged this hypothesis (Kataria et al. 2015). Several theories have been proposed to explain the putative prostatic cancer promoting role of phytanic acid. Among them is the suggestion that phytanic acid or its metabolites may be agonists for the peroxisome proliferator-activated receptor δ (PPARδ), which promotes cancer, or the overexpression of Amacr may allow switching of energy sources to fatty acid β-oxidation thereby facilitating production of reactive oxygen species, oxidative stress and DNA damage and cancer progression. Understanding the Amacr mechanism will aid in the design of drugs to treat cancers with Amacr overexpression. Some studies using Amacr inhibitors, immunotherapy or reduction of Amacr protein levels using siRNA have been conducted (Lloyd et al. 2013).

### 2.3.4 Amacr-deficient mouse model

The Amacr-deficient mouse was generated by disrupting the *Amacr* gene using homologous recombination where exons 1–3 and introns 1–2 were replaced with the neomycin-resistance gene. The breeding of heterozygous *Amacr*+/- mice represented a normal Mendelian distribution, which suggests the normal intrauterine and postnatal survival rate of *Amacr*−/− offspring. On a regular chow diet Amacr-deficient mice showed few pathological symptoms. The fertility of Amacr-deficient male and female mice was normal and their growth and body weights were comparable to age- and sex-matched controls. (Savolainen et al. 2004).

Like in the human deficiency, Amacr-deficient mice accumulated C27 bile acid intermediates and the level of mature C24 bile acids was decreased. This was accompanied by changes in gene expression of certain lipid metabolizing enzymes including some CYP enzymes possibly involved in ω-oxidation. It was speculated that the residual C24 bile acids in Amacr-deficient mice were produced via an Amacr independent manner through oxysterols and Mfe-1. (Savolainen et al. 2004).
The serum concentrations of phytanic and pristanic acids were not elevated in Amacr-deficient mice fed regular laboratory chow with a low phytol content, which indicates that the regular chow is a therapeutic diet for these mice. The decline in the bile acid pool did not affect intestinal triacylglycerol absorption, as the fatty acid contents of the stools were similar in Amacr-deficient and control mice. This was the case even when the mice were exposed to a triolein-enriched diet. No steatorrhea was observed. Some human Amacr-deficient patients have been suggested to suffer from lipid absorbent vitamin K-deficiency due to an elevation of the values in a prothrombin assay (Van Veldhoven et al. 2001). This was not seen in Amacr-deficient mice. These findings indicate that Amacr-deficient mice have no generalized defect in lipid absorption, which may be explained in part by the reduced but still sufficient amount of mature C24 bile acids and in part by the substitution of bile acid function with C27 bile acid precursors. (Savolainen et al. 2004).

The experiment conducted with the male mice, a diet enriched with phytol resulted in a disease state in Amacr-deficient mice but not in control mice. During a six-week observation period, one out of seven Amacr-deficient mice died already on day 9. The rest of the mice survived but they weighed 10% less than control mice after a phytol feeding period. The food intake did not change between the animals on different diets. During the 6-week phytol diet, Amacr-deficient mice lost 62% of the weight of their epididymal fat pads. Phytol feeding caused hepatomegaly and liver damage in Amacr-deficient mice. Histological examination of liver tissue by light microscopy revealed lobular accumulation of lymphocytes and Kupffer cells, multivacuolar degeneration and coagulation necrosis. Vacuolization was not due to accumulation of lipids examined with oil red O staining of frozen liver sections. In other tissues from mice fed the normal or the phytol enriched chow (liver, kidney, skeletal and heart muscles, lung, spleen, eye and brain) no morphological differences were found by light microscopy. Electron microscopy of liver specimens showed proliferation of smooth endoplasmic reticulum and peroxisomes, which were enlarged and unevenly shaped. The morphological changes of peroxisomes were not due to proliferation of peroxisomes. This was tested by feeding mice with clofibrate, which is a known proliferator of peroxisomes (Lazarow & De Duve 1976). Liver damage of Amacr-deficient mice on the phytol enriched diet was also observed in the serum where the concentration of alkaline phosphatase (APHOS) was elevated. In Amacr-deficient mice on the normal diet as well as on the phytol diet, the concentration of alanine amino transferase (ALAT) was unchanged. The one
Amacr-deficient mouse that died after 9 days was an exception having clearly higher concentrations of both APHOS and ALAT. Phytol feeding also resulted in lower concentrations of palmitic, oleic and stearic acids in the serum of Amacr knockout mice. (Savolainen et al. 2004).

Amacr-deficient human patients suffer from sensory motor neuropathy with upper motor neuron signs in legs (Ferdinandusse et al. 2000a). This was also tested in mice on normal and phytol diets using the hanging and grid tests, but no disturbance in muscular performance was noticed in Amacr-deficient mice. The acute toxic effects on Amacr-deficient mice seen in the phytol feeding experiment were assumed to be mediated by phytol metabolites phytanic and pristanic acids that accumulated in the body of Amacr-deficient mice. (Savolainen et al. 2004).

An interesting finding was that the serum cholesterol concentration of Amacr-deficient mice was reduced to about half of that found in control mice both on regular and on phytol diets. On the regular chow diet, the concentrations of triacylglycerols were the same in both genotypes but on a phytol diet, the concentration of triacylglycerols in Amacr-deficient mice was reduced 52% compared to the values in control mice. The lower cholesterol concentration was speculated to result from accelerated channeling of cholesterol towards bile acids or a possible isolated defect in cholesterol absorption. (Savolainen et al. 2004). In the literature one patient is reported to have a reduced serum cholesterol concentration (McLean et al. 2002).

### 2.4 Peroxisomal multifunctional enzymes

The two peroxisomal multifunctional enzymes have overlapping substrate and catalytic specificities but they share no sequence similarity, have separate evolutionary origins and are structurally very different. The N-terminal part of MFE-1 contains the enoyl-CoA hydratase and $\Delta^2\Delta^3$-enoyl-CoA isomerase activities. Isomerase activity is necessary for the oxidation of unsaturated fatty acids (Palosaari & Hiltunen 1990, Palosaari et al. 1991). The C-terminal part contains the 3-hydroxyacyl-CoA dehydrogenase activity (Kiema et al. 2002). In MFE-2 the 3-hydroxyacyl-CoA dehydrogenase activity is localized in the N-terminal part of the protein. The central part contains the enoyl-CoA hydratase activity and the C-terminal domain contains sterol carrier protein 2 activity (Qin et al. 1999). Both of these multifunctional enzymes can react with straight chain fatty acid enoyl-CoAs. In particular MFE-2 is thought to be responsible for the degradation of CoA esters of 2-methyl branched fatty acids and steroid derived
fatty acids in the bile acid synthesis pathway (Dieuaide-Noubhani et al. 1997). The discussion below focuses on the role of MFE-1 related to bile acid synthesis.

### 2.4.1 MFE-1

MFE-1 has broad substrate specificity. It accepts short and long chain enoyl-CoA esters as well as dicarboxylic acyl-CoA esters (Ferdinandusse et al. 2004, Nguyen et al. 2008). The crystal structure of rat MFE-1 bound to 2E-methylbut-2-enoyl-CoA demonstrates that MFE-1 can bind 2-methyl branched acyl groups (Kasaragod et al. 2013).

In MFE-2-deficiencies (discussed in section 2.1.4) it has been previously suggested that MFE-1 and Amacr serve as an alternative route for bile acid synthesis from 24S-hydroxy-oxysterols. The hydratase of MFE-1 is able to catalyze the hydration of 24E-DHCA/THCA-CoA to the 24S,25S-OH-DHCA/THCA-CoA, but this substrate is accepted neither by the dehydrogenase part of MFE-1 nor MFE-2. However, it is a substrate for Amacr producing 24S,25R-OH-DHCA/THCA-CoA, which is accepted by the MFE-1 dehydrogenase (Cuebas et al. 2002, Savolainen et al. 2004). The combined action of MFE-1 and Amacr in peroxisomal bile acid synthesis is supported by the observation that MFE-1 is up-regulated in mice having either MFE-2 or Amacr knocked out (Baes et al. 2000, Savolainen et al. 2004). Potential 24S,25R-substrates for MFE-1 can arise also in an Amacr independent manner through hydroxylations of oxysterols by at least two other enzymes. CYP3A4, which corresponds to CYP3A11 in mice, catalyzes 24S-hydroxylation of oxysterols generating 24S,25R-metabolites (Honda et al. 2001). In previous studies with Amacr-deficient mice expressing residual C24 bile acids, the gene expression of Cyp3a11 was moderately increased in liver, which supports the idea of an Amacr-MFE-2 independent bile acid synthesis pathway (Savolainen et al. 2004). Alternatively, 24S,25R-metabolites can be formed Amacr independently by cholesterol 24-hydroxylase (Cyp46a1), which eliminates cholesterol from the brain (Bjorkhem et al. 2001).

Peroxisomal MFE-1 is a monomeric protein that has a high degree of similarity with the α-chain of the mitochondrial trifunctional protein (Uchida et al. 1992). Recently it was shown that a mutation in one allele of MFE-1 gene encodes a MFE-1 variant that is mistargeted to mitochondria. This variant interferes with the assembly and function of the trifunctional protein with the outcome of Fanconi’s syndrome (Klootwijk et al. 2014). The expression of MFE-
1 in rodents is induced by peroxisome proliferators and other biological ligands that are recognized by the PPARα (Jia et al. 2003, Reddy & Hashimoto 2001).

### 2.4.2 Mfe-1-deficient mouse model

MFE-1-deficiency has not been reported in humans. In contrast to the severe phenotypes of Mfe-2 knockout and Mfe-1/Mfe-2 double knockout mice, mice deficient in Mfe-1 are viable and fertile and exhibit no detectable phenotypic defects. In Mfe-1 knockout mice, the response to PPARα activation was normal except for the reduction of peroxisomal proliferation. The lack of a gross phenotype is explicable in the presence of Mfe-2 (Qi et al. 1999). Bile acid synthesis was also normal in Mfe-1-deficient mice (Ferdinandusse et al. 2005). The breakdown of both saturated medium chain (Dirkx et al. 2007) and polyunsaturated long chain dicarboxylic acids (Nguyen et al. 2008) is impaired. The recent study in which Mfe-1-deficient mice were fed with coconut oil containing medium chain fatty acids to induce the activity of CYP4A (ω-oxidation gene) and the resulting increase in dicarboxylic acid production showed that MFE-1-deficiency leads to intracellular dicarboxylic acid accumulation, inflammation, fibrosis, and liver failure. These results highlight the importance of MFE-1 in the metabolism of medium chain fatty acids (Ding et al. 2013).

Mfe-2-deficient mice exhibit a severe postnatal growth retardation that leads to the premature death of 30–40% of the pups during the first weeks of life. The phenotype of Mfe-1/Mfe-2 double knockout mice was more severe and almost none of them survived beyond weaning because of a complete block at the level of second step in peroxisomal β-oxidation (Baes et al. 2002). Both Mfe-2 knockout mice and Mfe-1/Mfe-2 double knockout mice accumulated large amounts of C27 bile acid precursors in their liver, bile and plasma with a simultaneous reduction in mature C24 bile acids (Ferdinandusse et al. 2005).
3 Outlines of the present study

Lipids with methyl branched carbon chains arise endogenously or can enter into the body with food and be absorbed via the alimentary tract. These compounds are chiral long chain hydrophobic lipids and their excretion out of the body typically requires their metabolism to shortened, more hydrophilic compounds. The excretion takes place either after oxidation of the carbon skeleton all the way to carbon dioxide or excretion via bile as conjugated or free metabolites.

Amacr-deficient mice have been generated previously, and biochemical analyses showed that their lipid metabolism was greatly disturbed. In contrast to patients with AMACR-deficiency, the deficient mice are clinically symptomless under standard laboratory conditions (Savolainen et al. 2004). This discrepancy prompted us to hypothesize that the Amacr enzyme might have multiple roles in the lipid metabolism of mammals. To tackle this working hypothesis three experimental approaches were chosen as follows:

1. To analyze readjustment of cholesterol and bile acid metabolism in Amacr-deficient mice.

2. To elucidate the contribution of peroxisomal Mfe-1 to bile acid synthesis in mice.

2. To study the role of Amacr in the disposal of α-methyl branched lipids other than bile acid intermediates.
4 Materials and methods

Detailed descriptions of the materials and methods used can be found in the original articles referred to by their Roman numerals (I–III).

4.1 Animal care (I, II, III)

The use of mice in animal experiments was approved by the University of Oulu Committee on Animal Experimentation. Animal experimentation was done in the germ-free facilities of the Laboratory Animal Center, University of Oulu or in the animal facility of the Department of Biochemistry, University of Oulu. Mice were housed in a temperature-controlled room with equal periods (12 h) of darkness and light and provided with unrestricted access to water and standard rodent chow (Lactamin AB and Harlan Laboratories). At the starting point of the experiments the mice used were 3–4 months old adult mice. Before blood collection and tissue harvesting the mice were anesthetized and subsequently sacrificed by cervical dislocation. The feeding experiment was done by stressing mice with chow supplemented with 0.5% (w/w) phytol (Fluka). Experiments were done with male mice except aging tests on normal and phytol supplemented chow where both sexes were used. The condition of the mice was monitored daily by the staff of the animal facility. The consumption of chow and body weights were checked regularly.

4.2 Mouse models (I, II, III)

Single knockout mice with Amacr- and Mfe-1-deficiencies were obtained and genotyped with PCR as described previously (Qi et al. 1999, Savolainen et al. 2004). Both strains were in a C57BL/6 genetic background.

4.2.1 Generation of Amacr- and Mfe-1-deficient double knockout mice

Double knockout mice with Amacr- and Mfe-1-deficiency were obtained after two generations of breeding, starting from Amacr−/− and Mfe-1+− or Mfe-1−/− mice. Heterozygous Amacr+/− Mfe-1+/− mice were obtained and mated to generate the Amacr−/− Mfe-1−/− double knockout mouse line. Genotyping was based on PCR with primers for Amacr and Mfe-1 (Table 2).
Table 2. Sequences of primers used for PCR genotyping of mice.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amao-+/+</td>
<td>5´-ACCTTCCTGACTGTCACAAAGATAT-3´</td>
<td>5´-AAGCAAAAGGACCTTTCGCGAG-3´</td>
</tr>
<tr>
<td>Amao-−/−</td>
<td>5´-TCGCATTGCTAGGTGTCAC-T3´</td>
<td>5´-AAGCAAAAGGACCTTTCGCGAG-3´</td>
</tr>
<tr>
<td>Mfe-1+/+</td>
<td>5´-GAGCTGGCCTGGGGCTACACTA-3´</td>
<td>5´-TGAATGAAGCTGCGTTCCTTGACACCA-3´</td>
</tr>
<tr>
<td>Mfe-1−/−</td>
<td>5´-GAGCTGGCCTGGGGCTACACTA-3´</td>
<td>5´-TGAATGAAGCTGCGTTCCTTGACACCA-3´</td>
</tr>
</tbody>
</table>

4.3 Analysis of bile acids and fatty acids (I, II, III)

Bile acids and their derivatives were analyzed using an APEX II FT-ICR mass spectrometer controlled by Xmass 6.0.0 software and equipped with a 7T active shielded magnet and an APOLLO electrospray ion source (Bruker Daltonics). The internal standards used in negative ion mass spectra were pentadecanoic acid, heptadecanoic acid and/or lithocholic acid, glycolithocholic acid and tauroliothocholic acid in a nebulizing solvent of acetonitrile/10 mM ammonium acetate (1:1, v/v).

For determination of the total bile acid pool and composition (I), liver, gallbladder and small intestine were homogenized in four volumes (w/v) of 10 mM ammonium acetate, and an internal standard solution was added prior to recording the ion mass spectra. For the quantification of bile acid derivatives in liver (II, III), the liver tissue was homogenized in a nine-fold volume of phosphate buffered saline (PBS), insoluble material was removed by centrifugation and standard solution was added prior to lipid extraction as given (Savolainen et al. 2004). After evaporation, the extracts were re-dissolved in nebulizing solvent and the negative ion mass spectra were recorded. Bile fluids (II) of mice were diluted in water (1:10) and an aliquot was combined with nebulizing solvent and standard solution. The resulting mixture was used for the recording of negative ion mass spectra. For the determination of bile acid derivatives from serum (II), an aliquot of serum was mixed with water, standard solution and an aliquot of RP-18 (Macherey-Nagel). The resulting supernatant was removed and the residue was washed with water. Bile acids were then extracted and re-suspended in nebulizing solvent, and mass spectral data were recorded. For the determination of fecal bile acids (I), feces from individually housed mice were collected for 48 hours. Lyophilized ground feces with glycolithocholic acid and glycocholic acid in chloroform/methanol (1:1, v/v) as an internal standard solution were extracted. Evaporated extracts were re-suspended in
chloroform/methanol and diluted with nebulizing solvent and the negative ion mass spectra were recorded.

24S- and 24R-hydroxycholesterol concentrations from serum (II) were analyzed by LC-ESI-MS after enzyme-assisted derivatization for sterol analysis (EADSA) as previously described (Griffiths et al. 2013).

For the quantification of fatty acids in liver (III), the homogenized liver tissue was extracted with chloroform and methanol. The extract was mixed with 2.5 mM nonadecanoic acid methyl ester in methanol/chloroform (1/1, v/v), water and chloroform. After centrifugation, the lower phase was evaporated and redissolved and mixed in 1 M acetychloride in methanol and heated for 3 h at 74 °C. The resulting fatty acid methyl esters were extracted twice with hexane and after evaporation to dryness, the residue was dissolved in hexane. The fatty acid methylesters were separated by thin-layer chromatography (TLC) (Merck) using hexane/diethylether (7/3, v/v) as the developing solvent. The compounds were visualized in an iodine chamber. Areas containing fatty acid methylesters were scraped off and extracted with diethylether. The evaporated extract was redissolved in dichloroethane before the gas chromatographic analysis (CS Langerwehe) of fatty acids.

4.4 Lipid analysis (I)

Whole animal cholesterol concentration (I) was measured from ground and lyophilized mice. Fecal cholesterol (I) was measured from the lyophilized ground feces collected for 48 hours from individually housed mice. The lipids in these samples were extracted with the modified Folch extraction method and the cholesterol content was determined by an HPLC method using a Spherisorb Silica Column (Waters) with a PL-ELS 2100 evaporative light scattering detector (Polymer Laboratories) (Homan & Anderson 1998). The mouse liver lipids were also extracted with the Folch method (Folch et al. 1957). Total cholesterol (CHOL; Roche/Hitachi), triacylglycerols (TG; Roche/Hitachi), free cholesterol (F-CHO; Daiichi Pure Chemicals) and phospholipids (S-PL; Daiichi Pure Chemicals) were measured with enzymatic photometric methods. The lipidomic analysis from mouse livers was done at Zora Biosciences (Espoo, Finland) with mass spectrometric analysis. A QTRAP® 5500 instrument (Applied Biosystems/MDS Analytical Technologies) equipped with a robotic nanoflow-ion source NanoMateHD (Advion) in both positive and negative modes was used.
The multiple precursor ion scanning method (MPIS) was performed as described previously (Ekroos et al. 2002).

4.5 Analysis of cholesterol absorption (I)

Intestinal cholesterol absorption was measured by a fecal dual-isotope method (Turley et al. 1994, Wang & Carey 2003). Slightly anesthetized mice were dosed intragastrically with a mixture of 2 μCi [5,6-3H] β-sitostanol and 1 μCi [4-14C] cholesterol (American Radiolabeled Chemicals) solubilized in olive oil. They were then housed individually in cages with wire mesh bottoms and stools were collected for 24 h. Lipids were extracted using a modified Folch extraction procedure and the radioactivity was quantified with a Wallac WinSpectralTM 1414 Liquid Scintillation counter (Perkin Elmer Life and Analytical Sciences). The percent cholesterol absorbed was calculated as previously described (Turley et al. 1994).

4.6 Analysis of cholesterol synthesis (I)

The rate of de novo cholesterol synthesis in liver was measured using a 3H2O incorporation assay (Jeske & Dietschy 1980). Mice were injected intraperitoneally with 40 mCi of [3H]-labeled water. One hour later, the blood was drawn under anesthesia from the retro-orbital sinus and plasma was separated by centrifugation. Upon necropsy, livers were harvested and then saponified in alcoholic KOH. The non-saponifiable lipids were extracted with isopropyl alcohol-hexane (2:3 v/v) and fractionated by silica gel TLC in n-hexane/diethylether/acetic acid (49:21:0.7, v/v/v). Cholesterol and cholesterol ester spots were identified by co-migration of known lipid standards under ultraviolet light by spraying the TLC-plate with fluorescent 1,6-diphenyl-1,3,5-hexatriene and the spots were scraped from the plate. Incorporation of radiolabel was determined by scintillation counting (Perkin Elmer Life and Analytical Sciences). The specific activity of body water and the rate of hepatic sterol synthesis were calculated as previously described (Turley et al. 1981).

4.7 Plasma and serum analysis (I, II, III)

Blood samples were collected from 12-hour fasted or non-fasted, anesthetized mice by orbital bleeding or terminal blood was drawn from the inferior vena cava
after sacrifice. Serum or plasma was separated by centrifugation within 15 to 30 minutes after blood sampling and storage on ice.

Blood chemistry analyses according to standard methods (II, III) for serum sodium, potassium, chloride, creatinine, albumin, glucose, urate, bilirubin, total cholesterol, triacylglycerols, alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, creatine kinase and lactate hydrogenase, were done in the clinical laboratory of the University Hospital of Oulu.

Total and hepatic lipase activities (I) were determined from post-heparin plasma fluorometrically (Confluolip™ Continuous Fluorometric Lipase Test, Progen Biotechnik). PLTP and Lecitin-cholesterol acyltransferase (LCAT) activities (I) from serum were measured in the laboratory of Docent Matti Jauhiainen, University of Helsinki as previously described (Jauhiainen & Dolphin 1986, Jauhiainen et al. 1993). Mouse apo-A-I (I) was quantified by sandwich enzyme-linked immunoabsorbent assay (ELISA) and mouse pre-β-HDL by 2-D immunoeluotrophoresis (van Haperen et al. 2000).

For serum lipoprotein profiles (I) plasma from five overnight fasted mice were pooled and fractionated using fast protein liquid chromatography (FPLC) (Superose™ 6 10/300 GL column and an ÄKTA™ Explorer 10 instrument, Amersham Pharmacia Biotech). The fractions were analyzed for cholesterol and triacylglycerols with enzymatic photometric methods (Roche Diagnostics).

### 4.8 Histological analysis (II, III)

For light microscopy analysis (II, III) samples from various tissues were fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Special stainings were used to distinguish connective tissue (Masson’s trichrome) in liver sections and myelin (Luxol fast blue) in brain sections. Immunohistochemical stainings were done from paraffin embedded liver and brain sections. Immunostaining was done with a commercial kit (ab64261 – Rabbit Specific HRP/DAB (ABC) Detection Kit, Abcam). Primary antibodies used for liver sections were a monoclonal rabbit antibody against F4/80 (Novus Biologicals) to detect macrophages and a polyclonal rabbit antibody against cytokeratin 7 (Novus Biologicals) to detect bile ducts. The primary antibody used for brain sections was a rabbit antibody against human glial fibrillary acidic protein (GFAP) (Novus Biologicals). Immunohistological sections were counterstained with Harris hematoxylin (Sigma-Aldrich). Samples
were examined with an Olympus BX51 light microscope equipped with Cell^M imaging software.

For transmission electron microscopy (II) liver samples were fixed in a 1% glutaraldehyde/4% formaldehyde mixture in 0.1 M phosphate buffer. After post fixation in 1% osmium tetroxide and dehydration in acetone, samples were embedded in Epon LX 112 (Ladd Research Industries) and thin sections were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems). Sections were stained in uranyl acetate and lead citrate and examined using a Philips CM100 transmission electron microscope equipped with a Morada CCD (charge-coupled device) camera (Olympus Soft Imaging Solutions).

4.9 Molecular biological analysis (I, II, III)

Gene expression analysis from mouse liver and intestine tissues were done by quantitative real-time PCR (I, II). For the analysis, intestinal RNA was isolated with RNeasy Mini Spin columns (Qiagen) and hepatic RNA with an RNeasy Mini Kit (Qiagen). cDNA was produced with a RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas). PCR was performed with a 7500 Real Time PCR System (Applied Biosystems) using fluorogenic probe-based TaqMan chemistry with a TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. TaqMan Gene Expression Assays for each studied gene were purchased from Applied Biosystems. For the relative quantification of gene expression, the results were normalized using glyceraldehyde-3-phosphate dehydrogenase (GADPH) as an endogenous control for each sample.

For microarray analysis (III) from liver tissue, the total RNA was isolated with TRIZol reagent according to the manufacturer’s specifications (Invitrogen). The transcriptional profiles of liver were probed using the whole genome Affymetrix GeneChip® Mouse Genome 430 2.0 Array. For both genotypes and diets, livers from 4 different mice were used. Labeling of the samples and the hybridizations, washing and scanning of the chips were carried out at the MicroArray Facility in Leuven (MAF, Leuven, Belgium). The expression data were analyzed with Chipster software (Kallio et al. 2011). The complete data are available under GEO record GSE40125 (Edgar et al. 2002).
5 Results

In previous studies, it was found that Amacr-deficient mice show no clinical symptoms under standard laboratory conditions despite the accumulation of C27 bile acid intermediates and low levels of primary and secondary C24 bile acids in bile, serum, and liver. Even the absorption of fatty acids was unchanged. However, the serum cholesterol levels were reduced in Amacr-deficient mice. In contrast, the short-term phytol enriched diet induced liver injury in Amacr-deficient mice. (Savolainen et al. 2004). In this study cholesterol and bile acid metabolism was studied to uncover the underlying adaptive mechanism that allows these mice to have a normal life span. The residual C24 bile acid pool in Amacr-deficient mice implies the existence of alternative pathways of bile acid synthesis. This option was studied using a double knock out mouse model deficient in Amacr and Mfe-1. Finally the effects of a prolonged phytol diet on Amacr-deficient mice and the molecular mechanism responsible for the effects by phytol were examined.

5.1 Clinical phenotype of Amacr\(^{-}/\)Mfe-1\(^{-}/\) double knockout mice (II)

Earlier studies have shown that under standard laboratory conditions mice with disrupted Amacr or Mfe-1 appeared normal. Also in Amacr\(^{-}/\)Mfe-1\(^{-}/\) double knockout mice there was no difference in appearance compared to wild type controls. Crossbreeding analysis with heterozygous Amacr\(^{+/-}\)Mfe-1\(^{+/-}\) double knockout mice produced littermates in all expected genotypes (Table S1 in II). Both male and female double mutant mice were viable and fertile, except that the average litter size of double nullizygous pairs was smaller (4.6 pups) compared with double heterozygous pairs (6.4 pups) (Figure 1A in II). The weight gain (Figure 1B in II) and life span (Figure 7 in II) of double knockouts were comparable to wild type controls. In this study the average weight of 3–3.5 months old Mfe-1\(^{-/-}\) single knockout male mice was 3.6 g heavier than wild type, whereas there was no size difference between wild type, Amacr\(^{-/-}\) and Amacr\(^{-/-}\)Mfe-1\(^{-/-}\) mice (Figure 1C in II). Liver weight was increased at 3–3.5 months of age in Amacr\(^{-/-}\) and Amacr\(^{-/-}\)Mfe-1\(^{-/-}\) mice compared to wild type and Mfe-1\(^{-/-}\) single knockout mice (Figure 1D in II).

During aging, Amacr\(^{-/-}\) and Amacr\(^{-/-}\)Mfe-1\(^{-/-}\) mice tended to develop liver tumors. The pathological analysis of livers of double knockout mice revealed oval cell hyperplasia, cholangitis, hepatocellular carcinoma or hepatocholangiocarcinoma at the age of 1–2 years potentially caused by the accumulation of C27 bile acid intermediates and low levels of primary and secondary C24 bile acids in bile, serum, and liver.
bile acid intermediates, which are considered to be toxic (Ferdinandusse et al. 2009a).

5.2 Bile acid analysis (I, II)

The total bile acid pool size was measured as the sum of the bile acids and bile acid intermediates in liver, gall bladder and small intestine. Because bile acids excreted in feces do not contribute any more to the metabolic active bile acid pool, they were not considered in this calculation. Compared to wild type control mice the bile acid pool size was reduced 69% in Amacr−/− single knockout and Amacr−/−Mfe-1−/− double knockout mice. The bile acid composition in these mice was changed from mature C24 bile acids to C27 bile acid intermediates and their taurine conjugates. Compared to wild type mice in which the main bile acid type was cholic acid along with its taurine conjugates and cholenolic acid, the amount of mature C24 bile acids was 14% in Amacr−/− single knock out and 17% in Amacr−/−Mfe-1−/− double knockout mice (Figure 1 in I and Table 1 in II). In Mfe-1−/− mice the size of the total bile acid pool was reduced by 32% compared to wild type mice. The reduction was mainly seen in unconjugated cholic acid. The main bile acids in both wild-type and Mfe-1−/− mice were mature C24 bile acids, and C27 bile acid intermediate levels were close to the detection limit (Table 1 in II).

As shown in Table 2 (modified from Tables S2, S3 and S4 in II), in bile fluid Amacr−/−Mfe-1−/− double knockout mice had a remarkably lower concentration of total bile acids and bile acid intermediates compared to wild type and single Mfe-1−/− mice (17% and 18%, respectively). In Amacr−/− mice the concentration was about 43% of that found in wild type mice. Amacr-deficiency changed greatly the ratio of C24 bile acids to C27 precursors as wild type and Mfe-1−/− mice with normal Amacr activity had about 300 times more C24 bile acids than C27 precursors in their bile fluid compared to Amacr−/− and Amacr−/−Mfe−/− mice. The mature bile acids in bile fluids of all genotypes were mostly taurine-conjugated, however Amacr−/−Mfe−/− mice had more unbound bile acids and less taurine-conjugated bile acids compared with wild type and Mfe-1−/− mice. Taurine-conjugated C27 bile acid intermediates in bile fluid of Amacr−/−Mfe-1−/− mice were decreased when compared to Amacr−/− mice, but increased when compared to wild type and Mfe-1−/− mice (Figure 2 in II). This indicates that breakdown of cholesterol is possibly via 24-hydroxylation, and that the dehydrogenase activity of MFE-1 can process their further degradation.
The size and composition of the hepatic bile acid pool in Mfe-1−/− mice did not differ from that of wild type mice (Table 2 and Table S3 in II). Liver of Amacr−/− mice contains equal amounts of C24 bile acids and greatly increased C27 bile acid intermediates when compared with the livers of wild type and Mfe-1−/− mice. Liver samples from Amacr−/+ Mfe−/− mice contained less C24 bile acids compared with the other three genotypes. The amount of hepatic C27 bile acid intermediates in double knockout mice is decreased when compared with Amacr−/− mice, but increased when compared with wild type and Mfe-1−/− mice.

In serum samples, the concentration of mature C24 bile acids did not differ between any of the genotypes studied. Again both Amacr−/− and Amacr−/+ Mfe−/− mice showed accumulation of C27 bile acid intermediates in serum compared with wild type and Mfe-1−/− mice (Table 3 and Tables S2–S4 in II). Of note, the standard deviations for serum samples are large.

Table 3. The concentrations of total bile acids in the bile fluid, in the livers, and in the serum of wild type, Mfe1−/−, Amacr−/−, and Amacr−/+ Mfe−/− mice.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Wild type</th>
<th>Mfe-1−/−</th>
<th>Amacr−/−</th>
<th>Amacr−/+ Mfe−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile fluid (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total free BA</td>
<td>7.70 ± 5.39</td>
<td>2.92 ± 0.68</td>
<td>20.3 ± 7.5</td>
<td>5.10 ± 3.93</td>
</tr>
<tr>
<td>Total T-BA</td>
<td>199 ± 48</td>
<td>200 ± 94</td>
<td>97.1 ± 18.7</td>
<td>31.0 ± 22.3</td>
</tr>
<tr>
<td>Total C24-BA</td>
<td>206 ± 53</td>
<td>202 ± 94</td>
<td>69.7 ± 15.9</td>
<td>19.0 ± 3.2</td>
</tr>
<tr>
<td>Total C27-BA</td>
<td>0.69 ± 0.23</td>
<td>0.71 ± 0.31</td>
<td>47.7 ± 10.4</td>
<td>17.1 ± 13.0</td>
</tr>
<tr>
<td>Total BA</td>
<td>207 ± 53</td>
<td>203 ± 94</td>
<td>117 ± 26</td>
<td>36.1 ± 26.2</td>
</tr>
<tr>
<td>C24/C27 Total BA</td>
<td>300.5</td>
<td>283.7</td>
<td>1.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Liver (µmol/soluble supernatant from 1 kg of homogenized liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total free BA</td>
<td>95.5 ± 79.5</td>
<td>114 ± 22</td>
<td>172 ± 77</td>
<td>59.9 ± 16.3</td>
</tr>
<tr>
<td>Total T-BA</td>
<td>8.88 ± 3.95</td>
<td>12.7 ± 3.8</td>
<td>139 ± 76</td>
<td>23.7 ± 10.3</td>
</tr>
<tr>
<td>Total C24-BA</td>
<td>104 ± 83</td>
<td>125 ± 26</td>
<td>118 ± 70</td>
<td>33.4 ± 13.5</td>
</tr>
<tr>
<td>Total C27-BA</td>
<td>0.51 ± 0.22</td>
<td>1.21 ± 0.48</td>
<td>194 ± 83</td>
<td>50.2 ± 13.2</td>
</tr>
<tr>
<td>Total BA</td>
<td>104 ± 83</td>
<td>126 ± 26</td>
<td>312 ± 153</td>
<td>83.6 ± 26.7</td>
</tr>
<tr>
<td>C24/C27 Total BA</td>
<td>203.3</td>
<td>103.9</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Serum (µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total free BA</td>
<td>3.41 ± 2.25</td>
<td>19.4 ± 15.2</td>
<td>12.7 ± 9.6</td>
<td>7.25 ± 3.37</td>
</tr>
<tr>
<td>Total T-BA</td>
<td>13.4 ± 16.1</td>
<td>23.5 ± 16.7</td>
<td>2.42 ± 1.05</td>
<td>5.74 ± 4.89</td>
</tr>
<tr>
<td>Total C24-BA</td>
<td>16.4 ± 18.2</td>
<td>42.5 ± 31.8</td>
<td>5.23 ± 4.75</td>
<td>3.80 ± 2.99</td>
</tr>
<tr>
<td>Total C27-BA</td>
<td>0.35 ± 0.10</td>
<td>0.41 ± 0.13</td>
<td>9.89 ± 5.93</td>
<td>9.19 ± 5.27</td>
</tr>
<tr>
<td>Total BA</td>
<td>16.8 ± 18.3</td>
<td>42.9 ± 31.9</td>
<td>15.1 ± 10.7</td>
<td>13.0 ± 8.3</td>
</tr>
<tr>
<td>C24/C27 Total BA</td>
<td>47.0</td>
<td>103.2</td>
<td>0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Values represent means ± sd.
The potential role of an Amacr independent pathway via Mfe-1 to synthesize bile acids was further examined by determining the concentrations of 24S- and 24R-hydroxycholesterol concentrations in the serum. There were no statistically significant differences in the concentration of free 24S-hydroxycholesterol between different knockout lines and wild type mice. In wild type mice the concentration of 24R-hydroxycholesterol was about one fifth of the level of the 24S-isomer and the levels were similar in all knockout mouse models. The levels of the 25R-isomer of C27 bile acid intermediates 7α-hydroxy-3-oxocholest-4-enoic acids were significantly higher in both the Amacr−/− and Amacr−/−Mfe-1−/− mice compared to wild type control and Mfe-1−/− mice. On the other hand, the 25S-isomer was absent from the Amacr−/− and Amacr−/−Mfe-1−/− mice, but present at low levels in the control and Mfe-1−/− mice (Table 2 in II).

In Amacr−/− mice the amount and composition of bile acids excreted in feces were also analyzed (I). The excretion of bile acids was doubled in Amacr−/− mice (Figure 2F in I) and the fecal bile acid composition was profoundly altered when compared with wild type mice (Figure S1 in I). Wild type mice excrete mainly deoxycholic acid and cholic acid in addition to some sulfated cholic acid. These bile acids are almost absent in the feces of Amacr−/− mice and instead they excrete large amounts of the sulfated and unconjugated C27 bile acid precursors THCA and DHCA.

5.3 Cholesterol balance studies (I)

Amacr-deficiency in mice results in a decrease in the total bile acid pool size and a change of bile acid composition from mature C24 bile acids to C27 bile acid intermediates. The changes in the bile acid pool and profile decreased the intestinal cholesterol absorption 16% in Amacr−/− mice compared to wild type controls (Figure 2A and 2B in I). The expression of selected genes involved in cholesterol absorption in duodenum and proximal jejunum, which are the major sites for intestinal cholesterol absorption, was also studied. No significant change was seen in the expression levels of the Npc1l1 transporter needed for cholesterol absorption or in the expressions of Abcg5 and Abcg8 transporters involved in cholesterol secretion into the lumen. (Figure 6A in I).

The excretion of cholesterol in feces was 48% higher in Amacr−/− mice compared to wild type mice (Figure 2C in I). Still the total body cholesterol pool was the same between genotypes (Figure 2D in I). In Amacr−/− mice the cholesterol balance in the body was maintained by inducing the endogenous
cholesterol synthesis rate, which was 5-times higher compared to wild type control mice (Figure 2E in I). The gene expression analysis from liver tissue with quantitative real-time PCR showed elevated expression levels of genes involved in cholesterol synthesis such as 3-hydroxy-3-methylglutaryl-CoA reductase (Hmgcr) and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (Hmgcs1) in Amacr−/− mice (Figure 7 in I). The expression of those genes is normally activated via processed and nucleus-targeted Srebp2. Insulin induced gene 2 (INSIG2) is a well-known oxysterol and cholesterol sensor that affects proteolytic activation of SREBPs thus preventing SREBP2 maturation. However, our qPRC data revealed no change in the expression of Insig2 or Srebp1, and the 1.65-fold elevation of Srebp2 expression did not reach statistical significance (p = 0.06) (Figure 7 in I).

Due to the increased cholesterol synthesis in Amacr−/− mice, levels of hepatic cholesterol, triacylglycerols and phospholipids were measured. Amacr deletion resulted in a slight, but statistically significant decrease in cholesterol level in liver (Figure 5A in I). At the same time, hepatic levels of triacylglycerols and phospholipids were not changed (Figure 5B and 5C in I). Hepatic phosphatidylcholine (PC) and lysophosphatidyl choline (LPC) were increased in Amacr−/− mice (Figure S2 in I). These results indicate that although bile acid and cholesterol metabolism is significantly affected in Amacr−/− mice, the lipidomic status in liver remains almost unchanged.

### 5.4 Effect of phytol diet on Amacr−/− mice (III)

Previous studies, where Amacr−/− mice were fed with a diet supplemented with 0.5% phytol, showed that phytol treated Amacr−/− mice developed liver injury during the six-week feeding period. In the current study the feeding period was extended as long as the mice tolerated it. A considerable loss of body weight (more than 10%) or decrease in physical activity was chosen as the end-point criteria for the experiment. The first Amacr−/− mouse became moribund after two weeks on the phytol diet. Within 36 weeks all Amacr−/− mice (n=12) reached the end-point on the phytol diet, while more than 90% of wild type (n=16) still thrived (Figure 2A in III). On the normal laboratory chow diet the life span of Amacr−/− mice is normal (Figure 5 in II). About one third of the Amacr−/− mice on the phytol diet had rapid weight loss near the end-point, loss of one third of the body weight combined with slow movement. The rest of Amacr−/− mice were sacrificed due to slow or no movement. There was no correlation between the time the animal tolerated phytol and the symptoms at the end-point. Also no
differences in weight gain between wild type and Amacr−/− mice were noticed during the diet before the end-point (Figure 2B in III). The shorter two weeks phytol diet did not result in a difference in body weights between wild type and Amacr−/− mice (Figure 5A in III), but already after two weeks on the phytol diet the liver weight/body weight ratio in Amacr−/− mice was greater than in wild type mice indicating hepatomegaly (Figure 5B in III).

The motor coordination and muscular performance of the mice was tested during the phytol diet at weeks 6, 10, 12, 18, 24 and 30 by subjecting them to the hanging and grid tests. In the hanging test the animal should reach the end of the 50 cm thread in the 1 minute time limit and in the grid test hold on to the grid for 1 minute. No evident differences in these tests were seen between wild type and Amacr−/− mice on control or phytol diets analyzed by chi-square tests (Tables 1 and 2 in III).

After two weeks of the phytol diet hepatic lipid levels were also analyzed. There were no significant differences in the amount of straight chain, saturated or unsaturated fatty acids between wild type and Amacr−/− mice livers. The accumulation of branched chain fatty acids was seen both in the livers of wild type and Amacr−/− mice after two weeks phytol diet, but as expected, the increase was much higher in Amacr−/− mice. The main branched chain fatty acids that accumulated after a phytol diet in Amacr−/− mice were phytanic and pristanic acids (Figure 5C in III). In addition, TMTD and TMUD, the shorter methyl branched intermediates of phytol breakdown, accumulated slightly in Amacr−/− mice after phytol treatment (Figure 5D in III). Exposure of Amacr−/− mice to phytol amplifies changes in bile acid composition (Table 4 in III). In wild type mouse liver, the total bile acid content decreases to 71% after two weeks on a phytol enriched diet. In contrast, in Amacr−/− mouse liver, the amount of free C27-bile acid intermediates is doubled but the amount of taurine-conjugated C27-bile acid intermediates decreased to 10% of the levels in Amacr−/− mice on a control diet.

5.5 Gene expression analysis (I, II, III)

The successful inactivation of the Amacr gene was demonstrated in Amacr−/− and Amacr−/−Mfe-1−/− mice with quantitative real-time PCR analysis, where no expression of Amacr was detected, and no difference in the expression of Amacr between wild type and Mfe-1−/− mice was seen. Expression of Mfe-1 was clearly reduced in Mfe-1−/− and Amacr−/−Mfe-1−/− mice. The low, but measurable
expression signals were presumably due to the fact that in these mice exon 4 is replaced by a knockout cassette and primers in qPCR studies bind to the exon-exon boundary between exons 2 and 3, thus enabling detection of weak signal in qPCR (Figure 6 in II). On the protein level no MFE-1 has been detected in Mfe-1−/− mice studied by Western blot analysis (Qi et al. 1999).

In addition to qPCR analysis of selected genes of Amacr−/− mice in intestinal cholesterol absorption and hepatic cholesterol synthesis reported in chapter 5.3, transcription factors involved in cholesterol and bile acid metabolism were also studied from intestinal tissue. The expression of Lxra, Lxrβ, Fxr and Ppara appeared to be unchanged. The expression of Slc10a2 encoding the apical sodium-dependent bile acid transporter ASBT/IBAT was increased in the ileum of Amacr−/− mice (Figure 6B in I). In the intestine ASBT is thought to be the major route for active bile acid uptake in the enterohepatic circulation. In Amacr−/− mice the ileum section responds to the lack of bile acids by enhancing their uptake via ASBT. FGF15 is found in the absorptive cells of the mouse ileum and plays an important role in feedback inhibition of hepatic bile acid synthesis (Inagaki et al. 2005). The expression of Fgf15 in the distal ileum of Amacr−/− mice was decreased (Figure 6C in I).

Quantitative real-time PCR was also adopted to reveal how loss of MFE-1, Amacr or both activities affect the genes mediating the peroxisomal steps of side-chain shortening in bile acid synthesis at the molecular level. The hepatic expression of genes needed to activate bile acid intermediates to the corresponding CoA-thioesters, such as Slc27a5 or Slc27a2 was similar in all genotypes. In mouse the Amacr-deficiency causes up-regulation of genes downstream in the β-oxidation pathway such as Acox2 and Mfe-2, but not Scpx, whereas in Amacr−/−Mfe-1−/− mice, only Acox2 expression is increased. In single Mfe-1−/− knockout mice, no gene expression changes were seen. The expression of Baat, the bile acid conjugating enzyme, was up-regulated in Amacr−/− mice but not in other mutant genotypes (Figure 6A in II).

The bile acid synthesis pathway through oxysterols in an Amacr independent manner requires 24-hydroxylation to generate 24S-OH,25R-DHCA/THCA-CoA substrate for MFE-1. At least two enzymes exist in mice to make these intermediates, namely cholesterol 24-hydroxylase CYP46A1 and CYP3A11. The expression of these genes was tested with qPCR, and in Mfe-1−/− and Amacr−/−Mfe-1−/− mouse liver, expression of these genes was similar to that of wild type mice. The expression of Cyp3a11 was moderately increased and the expression of Cyp46a1 was decreased in livers of Amacr−/− mice (Figure 6B in II).
which confirmed the previous similar findings by Savolainen et al. (2004). Together with the increased expression of Cyp3a11 and Mfe-1 in Amacr−/− mice, this supports the idea that MFE-1 participates in bile acids synthesis independently from Amacr.

Gene expression analysis with a microarray study from liver samples of male mice was also performed. When comparing gene expression from wild type and Amacr−/− mice on standard laboratory chow, Amacr-deficiency caused statistically different expression changes in 44 genes. Those genes were mainly involved in cholesterol, steroid and isoprenoid biosynthesis pathways (Table 5 in III), which supports the finding of induced cholesterol synthesis described in chapter 5.3.

The effect of the phytol diet on gene expression in liver of wild type and Amacr−/− mice was studied by microarray analysis. Feeding wild type mice with phytol affected the expression of 171 genes when compared to wild type mice fed a control diet. The most affected pathways according to Gene Ontology were related to lipid metabolism, response to foreign substances and circadian clock (Table 6 in III). When studying the effect of the phytol diet on the livers of Amacr−/− mice, the expression of 87 genes was changed in a statistically significant manner. Those genes were mostly related to oxidation-reduction, cell division and inflammatory response pathways (Table 7 in III).

5.6 Histological analysis (II, III)

Light microscopic analysis of hematoxylin and eosin stained mouse liver samples showed that disruption of Amacr does not change the histology at the age of 3–3.5 months compared to wild type mouse livers. Previously, in Mfe-1−/− mice, liver histology at the age of 5 weeks was comparable to that of wild type mice (Jia et al. 2003). In this study we noticed degeneration especially in portal areas of Mfe-1−/−, Amacr−/− and in Amacr−/−Mfe-1−/− mouse livers at the age of 3–3.5 months (Figure 3 in II). Electron microscopy of the same liver specimens showed that overall, hepatocytes in Mfe-1−/−, Amacr−/− and in Amacr−/−Mfe-1−/− mice were round in shape compared with hepatocytes from wild type mice. Proliferation of peroxisomes was only detected in Amacr−/−Mfe-1−/− mouse hepatocytes. The space of Disse between liver sinusoidal endothelial cells and hepatocytes was enlarged in Amacr−/− mice (Figure 4A–4D in II). In Amacr−/−Mfe-1−/− mice the space of Disse was disrupted and also hyperplasia of smooth endoplasmic reticulum was detected. Mfe-1−/− mice had dilated bile canaliculi, whereas this feature was not so obvious in Amacr−/−Mfe-1−/− mice (Figure 4E–4H in II).
Additionally some collagen bundles were detected in the space of Disse in both Amacr−/− and Mfe-1−/− single knockout mice but fibrosis was not confirmed in Masson’s trichrome staining.

The histological analysis of spleen, pancreas and heart of Amacr−/− mice at the end-point after phytol feeding showed no abnormalities. Liver histology showed multifocal multilobular vacuolization mostly around the central veins (Figure 3A–3D in III). Moreover, multifocal hepatocyte necrosis mostly in the periportal area was seen in the livers of phytol fed Amacr−/− mice. Fibrosis was confirmed with Masson’s trichrome staining (Figure 3E–3H in III). Accumulation of fat laden Kupffer cells was observed in the centrilobular zone. This finding was verified by immunohistochemistry using the F4/80-antibody (Figure 4A–4D in III). Regenerative processes in the liver of Amacr−/− mice manifested as oval cell hyperplasia and bile duct proliferation. Bile duct proliferation was confirmed with immunohistochemical staining using a cytokeratin 7 antibody (Figure 4E–4H in III).

Histological analysis of kidney after phytol feeding showed dilated Bowman’s space containing exudate and atrophied glomeruli in some of the Amacr−/− mouse (6 A–6D in III). There was also multifocal loss of glomerulae and varying degrees of degenerative changes in tubular epithelial cells of Amacr−/− mice fed a phytol diet for longer periods.

Amacr−/− mice on the phytol diet did not display clear neurological symptoms at least in hanging and grid tests, and the problems in moving at the end-point may be due to their very sick overall status. Histological analysis of the brain showed that phytol feeding induced demyelination changes in Amacr−/− mice. Luxol fast blue staining showed light blue discoloration of myelin containing areas in Amacr−/− mice as early as 40 days on the phytol diet, whereas demyelination changes were not observed in wild type fed with phytol up to 15 months of age (Figure 7A–7D in III). Immunohistochemical staining of astrocytes with GFAP antibody revealed reactive astrocytes possibly clearing the damaged myelin (Figure 7E–7H in III).

5.7 Plasma and serum analysis (I, II, III)

Earlier studies showed that the serum cholesterol levels in Amacr−/− mice were reduced compared to those of wild type mice (Savolainen et al. 2004). To understand the background for the low cholesterol levels in serum, lipoprotein profiles were assessed. Disruption of Amacr leads to a clear reduction of both
LDL and HDL cholesterol, which correlates well with the total serum cholesterol measurements (Figure 3A in I). Although triacylglycerol levels did not differ between groups, they were redistributed among lipoprotein classes in Amacr<sup>−/−</sup> mice. Triacylglycerol lipoprotein profiling demonstrated that most of the triacylglycerols were concentrated in the VLDL particles in Amacr<sup>−/−</sup> mice, whereas in wild-type mice the triacylglycerols were more evenly distributed between all lipoprotein fractions (Figure 3B in I). Total lipase activity and hepatic lipase activity from postheparin plasma were lower in Amacr<sup>−/−</sup> mice than in wild type mice (Figure 3C in I). Lipases hydrolyze triacylglycerols and thus the increase in VLDL triacylglycerols can be due to the decreased lipase activities, especially that of LPL. PLTP activity was decreased by 21% in Amacr<sup>−/−</sup> mice compared to wild type (Figure 4A in I) while LCAT activity and the amount of mouse apoA-I or the proportion of pre-β-HDL were equivalent (Figure 4B–4C in I). The amount of free cholesterol and the total cholesterol in serum were decreased by 24% in Amacr<sup>−/−</sup> mice while phospholipid levels were decreased by 16% (Figure 4D in I).

In view of the hepatomegaly and histological changes seen in liver, the serum concentration of liver marker enzymes ALAT, APHOS and ASAT were measured. On the normal laboratory diet all of these three enzymes were elevated in Amacr<sup>−/−</sup>/Mfe-1<sup>−/−</sup> double knockout mice, whereas ALAT and ASAT were increased in Mfe-1<sup>−/−</sup> mice and ALAT and APHOS in Amacr<sup>−/−</sup> mice (Figure 5 in II).

At the end-point of the phytol feeding experiment, blood was collected from the mice. Various parameters were analyzed from the serum. The results of the blood chemistry analyses are seen in Table 3 in III. The cholesterol concentration in serum from mice on the standard laboratory chow diet resembled that seen in earlier studies (Savolainen et al. 2004). In contrast to previous studies, the concentration of triacylglycerols was diminished to about half of the wild type concentration in this study. The phytol diet further reduced the values of cholesterol and triacylglycerols. On the normal diet the potassium level was 9% lower in Amacr<sup>−/−</sup> mice. Hypokalemia was even lower in Amacr<sup>−/−</sup> mice fed the phytol diet, whereas potassium levels in wild type did not change when exposed to phytol. Phytol also caused hypernatremia in Amacr<sup>−/−</sup> mice as the concentration of sodium was 5% higher when compared to wild type and Amacr<sup>−/−</sup> mice on a normal diet. The high creatinine concentration in Amacr<sup>−/−</sup> mice (29.0 μmol/L) on the phytol diet indicates kidney failure and supports the histological findings in the kidney. Markedly elevated levels of liver marker enzymes ALAT (6-fold),
APHOS (7-fold), ASAT (3-fold) along with 7.6 times higher total bilirubin concentration in $Amacr^{-/-}$ mice on the phytol diet compared to wild type mice on a phytol diet clearly confirmed the liver damage seen in liver histology. Liver dysfunction also caused a decrease in urate concentration of $Amacr^{-/-}$ mice (53.6 μmol/L) compared to wild type mice (103.5 μmol/L). Liver failure and kidney insufficiency probably lead to the severe hypoglycemia in phytol fed $Amacr^{-/-}$ mice.
6 Discussion

6.1 Metabolic adaptation in Amacr<sup>-/-</sup> mice

Amacr<sup>-/-</sup> mice accumulate C27 bile acid intermediates in their bile acid pool, whereas the levels of mature C24 bile acids are greatly decreased, which resembles the findings in AMACR-deficient patients. The total bile acid pool of Amacr<sup>-/-</sup> mice was reduced almost 70% compared to wild type control mice. Despite the markedly changed bile acid profile, Amacr<sup>-/-</sup> mice remain symptom free due to multiple metabolic adaptations.

In digestion, bile acids are crucial for the proper solubilization, digestion and uptake of dietary fats. Before the excretion of newly synthesized bile acids into bile, primary bile acids are conjugated with glycine or taurine by the peroxisomal enzyme BAAT. The conjugation increases the acidity and solubility of bile acids by decreasing their pK<sub>a</sub>, but at the same time decreases their membrane permeability (Alnouti 2009). Conjugation of C24 bile acids by BAAT is efficient but the conjugation is less efficient in the case of C27 bile acid intermediates (Ferdinandusse et al. 2009a). In the enterohepatic circulation the conjugated bile acids are efficiently reabsorbed in the distal ileum by ASBT while the unconjugated bile acids are absorbed by passive diffusion along the intestinal tract. Normally only about 5% of the bile acids are lost in feces. Amacr<sup>-/-</sup> mice have an increased fraction of C27 bile acid precursors that are less amidated by BAAT. This results in increased amounts of unconjugated bile acids. Since the ASBT have enhanced affinity towards glycine or taurine conjugated bile acids, the secretion of bile acids is increased in Amacr<sup>-/-</sup> mice. The distal ileum of Amacr<sup>-/-</sup> mice responds to the reduction of bile acids in the enterohepatic circulation by enhancing the gene expression of ASBT, which facilitates the uptake of bile acids. The secretion of bile acids was doubled compared to controls and large amounts of the secreted bile acids were sulfated and unconjugated DHCA and THCA. Indeed, sulfated bile acids excreted in feces can be another explanation for the increased bile acid excretion in Amacr<sup>-/-</sup> mice because, as shown in earlier studies, sulfated bile acids are poor substrates for ASBT (Craddock et al. 1998, De Witt & Lack 1980). According to earlier studies, C27 bile acids show dose-dependent cytotoxicity (Ferdinandusse et al. 2009a) and sulfation is considered to be a detoxifying mechanism for toxic bile acids and oxysterols (Alnouti 2009, Ren & Ning 2014).
It has been demonstrated in other mouse models with defects in bile acid synthesis, such as Cyp7A1, Cyp8B1 and Cyp27A1 knock out lines, that low levels of bile acids impair the absorption of cholesterol in the intestine and increase cholesterol excretion in feces (Erickson et al. 2003, Li-Hawkins et al. 2002, Repa et al. 2000, Schwarz et al. 1998). Similarly, Amacr−/− mice have decreased intestinal cholesterol absorption and enhanced cholesterol excretion in stool. An explanation for the reduced absorption of cholesterol could be diminished amounts of cholesterol transporters. However, the expression of genes encoding intestinal cholesterol transporters NPC1L1, ABCG5 and ABCG8 was not changed in Amacr−/− mice. These results indicate that most likely the decreased cholesterol absorption in Amacr−/− mice is the result of the reduced micellar solubilization caused by low levels of C24 bile acids in the intestine. This idea could be confirmed by cholic acid feeding where cholic acid rendered the bile acid pool more hydrophilic in Amacr−/− mice, which improved the micellar solubilization and cholesterol absorption. Such improvements have been seen in other knockout mouse lines mentioned above.

Despite the reduced cholesterol uptake via absorption and enhanced cholesterol output into feces as cholesterol and bile acids, the total cholesterol pool was unchanged in Amacr−/− mice. To maintain the cholesterol balance, the cholesterol synthesis pathway in the liver of Amacr−/− mice was induced 5-fold compared to control mice. The increased expression of genes in the cholesterol synthesis pathway such as Hmgcr and Hmgcs1 was shown to be responsible for the enhanced hepatic cholesterol synthesis in Amacr−/− mice. Similar activation has been demonstrated in other bile acid deficient mouse lines such as Cyb8b1, Cyp27a1 and Mfe−2 knockout mice (Li-Hawkins et al. 2002, Martens et al. 2008, Repa et al. 2000). The expression of HMGCR and HMGCS1 is activated via nucleus-targeted SREBPs when cholesterol levels are low. The proteolytic activation of SREBPs is prevented by INSIG 2 when cholesterol or oxysterol levels are high. In our study, the qPCR data revealed no change in the expression of Insig2 or Srebp1 in Amacr−/− mice. The expression of Srebp2 was 1.65-fold elevated but this finding did not reach statistical significance (p = 0.06). The results in paper III support the findings of induced cholesterol synthesis as we showed that the expression of genes involved in cholesterol biosynthesis was one of the most affected Gene Ontology pathways when comparing liver samples from wild type and Amacr−/− mice on the regular chow diet.

Lipidomic analysis from liver showed that Amacr-deficiency led to a slight, but statistically significant decrease in the cholesterol level in liver, but the levels
of triacylglycerols and phospholipids were not changed (Figure 5A in I). Hepatic PC and LPC, a derivative of PC, were increased in Amacr−/− mice. PC is a major and LPC is a minor constituent of cell membranes. A recent study shows that the administration of PC for chronic, active hepatitis resulted in a significant reduction of disease activity in mice (Niebergall et al. 2011). The increased hepatic PC and LPC in Amacr−/− mice might contribute to the finding that liver histology of Amacr−/− mice is fairly normal in early life.

An earlier study has shown that the serum cholesterol concentration in Amacr−/− mice was lower than in wild type control mice (Savolainen et al. 2004). A moderate reduction in serum cholesterol has been described also in an Amacr-deficient patient (McLean et al. 2002). In our study the level of free cholesterol and the total cholesterol in serum were decreased by 24% in Amacr−/− mice. Also phospholipid levels were decreased by 16%. As seen in the present study, Amacr-deficiency leads to reduction of both LDL and HDL cholesterol. The amount of apoA-I or pre-β-HDL did not differ between genotypes showing that lack of apoA-I is not responsible for the reduced HDL cholesterol. Instead, total and hepatic lipase and PLTP activities were reduced, which can explain in part the decreased systemic HDL-cholesterol levels. Normally, when LPL-facilitated lipolysis is active, PLTP enhances the transfer of post-lipolytic phospholipid enriched surface-remnants to HDL thus stabilizing or even increasing the serum HDL-pool (Jiang et al. 1999, Tall et al. 1985). LCAT participates in maturation of HDL particles by converting free cholesterol into cholesteryl esters, but the activity of LCAT in Amacr−/− mice was not changed. The expression levels of cholesterol receptors like scavenger receptor class BI (SR-BI) or LDLR along with its degrading proteins IDOL and PCSK9 would have been interesting to study to determine their contribution to lower serum cholesterol levels. Unfortunately, this was not performed in this study. The triacylglycerol levels were the same between animal groups, but they were redistributed among lipoprotein classes in Amacr−/− mice. The reduced lipase activity apparently resulted in increased VLDL-associated triacylglycerols but reduced LDL- and HDL-associated triacylglycerols.

Ligand-activated transcription factors like FXR, LXR and PPARs are important regulators of lipid metabolism in liver and intestine. Therefore, the expression of these transcription factors in Amacr−/− mice was examined. qPCR studies implemented previously (Savolainen et al. 2004) and in the present study revealed no changes in the expression levels of FXR, LXR or PPAR, although the amounts of their ligands were changed. The genetic defect in bile acid
synthesis in \textit{Amacr}^{-/-} mice decreases the amount of mature bile acids as FXR ligands. Since the expression of PLTP is regulated transcriptionally by FXR (Tu \\& Albers 2001, Urizar \textit{et al}. 2000), the decreased availability of bile acids in \textit{Amacr}^{-/-} mice leads to lowered PLTP expression and low serum PLTP activity. Attenuated FXR-signaling can also explain the higher expression level of \textit{Cyp7a1} in liver (Savolainen \textit{et al}. 2004). The expression of \textit{Fgf15} in the distal ileum of \textit{Amacr}^{-/-} mice was decreased. In contrast to the case in Amacr-deficient mice, the uptake of bile acids via ASBT in the distal ileum activates FXR increasing the expression of FGF15/19 (Inagaki \textit{et al}. 2005). The secretion of FGF15/19 into the portal blood from the intestine leads to its binding to the receptor FGFR4/β-Klotho on hepatocytes and mediates the down-regulation of \textit{Cyp7a1} expression and suppression of bile acid synthesis (Calkin \\& Tontonoz 2012). Another mechanism for the upregulation of \textit{Cyp7a1} expression occurs via LXRs. The highly elevated hepatic cholesterol synthesis and resulting elevated oxysterols would support this idea, but decreased levels of cholesterol in liver and profiling LXR-dependent gene expression from qPCR data (e.g. \textit{Abca1}, \textit{Srebp1}) (Savolainen \textit{et al}. 2004) lead to the conclusion that LXR-regulated gene expression plays a minor role.

In summary, although mature C24 bile acids play a central role in digestion, lipid absorption and metabolic signaling, \textit{Amacr}^{-/-} mice, in which the mature bile acid pool is reduced by 90%, seem to be clinically symptomless on standard laboratory diet and even when challenged with a high fat diet. Due to the disrupted feedback loop from mature bile acids, hepatic 7α-hydroxylation is accelerated with concomitant acceleration of cholesterol biosynthesis.

\subsection*{6.2 Role of Amacr and MFE-1 in bile acid synthesis in mice}

A mouse line deficient in both Amacr and Mfe-1 was generated to test the hypothesis that MFE-1 could participate in bile acid synthesis. This study confirmed that MFE-1 can contribute to the shortening of C27 bile acid intermediates in both Amacr dependent and Amacr independent pathways.

Studies in patients with a single gene deficiency in the bile acid synthesis pathway have shown that alternative pathways for bile acid synthesis must exist (Ferdinandusse \textit{et al}. 2001, Ferdinandusse \textit{et al}. 2006a, Setchell \textit{et al}. 2003). The knockout mouse models for the genes \textit{Amacr} and \textit{Mfe-2} both have decreased...
levels of mature bile acids with a concomitant increase in the levels of C27 bile acid intermediates (Baes et al. 2000, Savolainen et al. 2004). Despite a block in the peroxisomal β-oxidation pathway, both of these mouse models harbor a residual pool of mature C24 bile acids. These findings imply the existence of an alternative bile acid synthesis pathway. One alternative pathway could involve MFE-1 either with or without Amacr. This hypothesis was tested by generating a double knockout mouse model deficient in both Amacr and MFE-1 activities.

The physiological role of MFE-1 in peroxisomes has not been unveiled unambiguously due to its overlapping catalytic properties with MFE-2. No patients with isolated MFE-1-deficiency have been characterized so far hindering the discovery of the role of MFE-1. The importance of MFE-1 in the metabolism of medium chain fatty acids was discovered only recently (Ding et al. 2013). The overall course of reactions catalyzed by MFE-1 is similar to that of MFE-2, but the reactions occur through the opposite stereochernistry. The potential role for MFE-1 in bile acid synthesis was previously tested by determining \textit{in vitro} the ability of purified rat MFE-1 to accept bile acid synthesis intermediates as substrates. That study suggested that, together with Amacr, MFE-1 can participate in the generation of mature C24 bile acids. For example, 24E-DHCA/THCA-CoA can be hydrated by MFE-1 to 24S,25S-24-OH-DHCA/THCA-CoA, which after conversion by Amacr into the 24S,25R isomer can again serve as a substrate for the dehydrogenase unit of MFE-1 (Cuebas et al. 2002). Furthermore, the crystal structure of rat MFE-1 bound to 2E-methylbut-2-enoyl-CoA demonstrates that MFE-1 can bind 2-methyl branched acyl groups such as bile acid intermediates (Kasaragod et al. 2013).

The original studies of \textit{Mfe-1}−/− knockout mice did not reveal striking differences in the phenotype compared to wild type mice (Qi et al. 1999). However, MFE-2-deficiency in mice caused severe growth retardation leading to premature death of one third of the pups during the first weeks of life (Baes et al. 2000). Studies with \textit{Mfe1}−/−\textit{Mfe-2}−/− double knockout mice also exhibited postnatal mortality and a severe growth phenotype. These two mouse models with Mfe-2 depletion accumulated large amounts of C27 bile acid precursors in their liver, plasma and bile with a simultaneous reduction of mature C24 bile acids, whereas \textit{Mfe-1} knockout mice did not exhibit those changes (Ferdinandusse et al. 2005). Instead, in this study the total bile acid pool size in \textit{Mfe-1}−/− mice was decreased by 32% compared to wild type mice. The decrease was mainly due to the diminished amount of unconjugated cholic acid and to a lesser extent due to a decrease in the amount of chenodeoxycholic acid. This was not accompanied by
an increase in C27 bile acid intermediates as those are barely detectable in both Mfe-1−/− and wild type mice. These results indicate that MFE-1 can play a role in bile acid synthesis.

In this study no major differences between bile acid profiles of Mfe-1−/− and wild type mice were seen when bile acids were analyzed from bile fluid, liver and serum separately. Still the difference in the total bile acid pools obtained from liver compared with gall bladder and intestine was clear between Mfe-1−/− and wild type mice. A putative explanation is that MFE-1 could be an important player in intestinal bile acid metabolism. However, such a role of MFE-1 has thus far not been studied.

The bile acid composition of bile fluid, liver and serum in Amacr−/− mice differed from those in Amacr−/−Mfe-1−/− mice. The total amount of mature C24 bile acids in Amacr−/−Mfe-1−/− double knockout mice was much less than in the wild type and single knockout mice; in the bile fluid the amount was less than one-tenth of that found in wild type and Mfe-1−/− mice and less than one-third of the amount in Amacr−/− mice. The values from liver and serum samples followed the same trend. On the contrary, the amount of C27 bile acid intermediates in the Amacr−/−Mfe-1−/− double knockout mouse bile fluid was about 25-fold higher than the amount in wild type and Mfe-1−/− mouse bile fluid, but only 36% of the respective values in Amacr−/− mice. Similarly, the amounts of C27 bile acid intermediates in liver and serum samples followed the same trend. As discussed in chapter 6.1, the adjustment of cholesterol and bile acid metabolism in Amacr−/− mice by increasing hepatic cholesterol synthesis 5-fold has most likely allowed these animals to maintain a normal life span. This results in the accumulation of high levels of C27 bile acid intermediates in these mice. Presumably, the hepatic cholesterol synthesis rate in double knockout mice is not as high, thus levels of C27 bile acid intermediates are lower in the Amacr−/−Mfe-1−/− mice than in Amacr−/− mice. Overall, these results are in agreement with the hypothesis that Amacr independent bile acid synthesis via MFE-1 is also possible.

Our studies with Amacr−/− mice revealed that these mice still contained approximately 14% residual C24 bile acids, which indicates that mature bile acids can be synthesized in an Amacr independent process. The Amacr independent bile acid synthesis pathway via MFE-1 requires 24-hydroxylation that ultimately can lead to the generation of 24S-OH,25R-DHCA/THCA-CoA metabolites. In principle, 24S,25R-metabolites can be generated from cholesterol by cholesterol 24-hydroxylase (Cyp46a1) which is predominantly expressed in mammalian brain and is important for the elimination of cholesterol from the brain (Bjorkhem et al. 80
1998, Bjorkhem et al. 2001). The Cyp46a1 knockout mouse model did not show bile acid alterations, which indicates a minor role for CYP46A1 in bile acid synthesis (Lund et al. 2003). Another Amacr independent bile acid pathway can proceed via Cyp3a11 (corresponds to CYP3A4 in human), which catalyzes the 24S-hydroxylation of oxysterols to generate 24S,25R-metabolites (Honda et al. 2001). In this and in a previous study by Savolainen et al. (2004) it was shown that the expression of Cyp3a11 is moderately increased in the liver of Amacr−/− mice, whereas the expression of Cyp46a1 is decreased. Savolainen et al. (2004) also showed increased expression of Mfe-1 upon disruption of Amacr, which supports the involvement of Mfe-1 in bile acid synthesis. However, the mRNA levels of Cyp3a11 or Cyp46a1 were not altered in Mfe-1−/− and Amacr−/−Mfe-1−/− mice. Cyp3a11 and CYP3A4 have an important role in xenobiotic metabolism, but they have also been shown to be involved in cholesterol and bile acid metabolism (Inoue et al. 2011, Pikuleva 2006).

It was shown in this study that Amacr−/−Mfe-1−/− double knockout mice still have residual amounts of bile acids in their bile fluid (9%), serum (23%) and liver (32%) compared with wild type mice, which enable a normal lifespan in these mice. Those residual amounts can be produced by microsomal plus cytosolic pathways in which the side-chain shortening occurs without any peroxisomal enzymes (Ferdinandusse & Houten 2006). Because chenodeoxycholic acid is not formed via this pathway, total bile acid pool analysis reveals only trace amounts of dihydroxylated C24 bile acids in double knockout mice. Noteworthy of attention considering the survival of Amacr−/− and Amacr−/−Mfe-1−/− mice with minimal C24 bile acids is that C27 bile acids are the predominant bile acid type in some reptiles (Hofmann et al. 2010).

The defect in Amacr leads to significant accumulation of 25R-bile acid intermediates such as 7α-hydroxy-3-oxocholest-4-enoic acids in the serum of Amacr−/− and Amacr−/−Mfe-1−/− mice. The corresponding 25S-isomer was not detected in the Amacr−/−Mfe-1−/− and Amacr−/− mice, but was present at low levels in the serum of wild type and Mfe-1−/− mice. The same bile acid abnormality has also been detected in AMACR-deficient patients (Ferdinandusse et al. 2001, Setchell et al. 2003).

C27 bile acid intermediates are considered to be toxic (Ferdinandusse et al. 2009a) and their amounts are highly increased in Amacr−/− and Amacr−/−Mfe-1−/− mice. Hepatomegaly was seen in both of these genotypes. Yet, this did not influence the liver histology in light microscopic analysis of Amacr−/− mice at the age of 3-3.5 months. Previously, it has been seen that Mfe-1−/− liver histology at
the age of 5 weeks was similar to that of wild type mice (Jia et al. 2003). In our light microscopic analysis we noticed degeneration especially in portal areas of Mfe-1−/− and Amacr−/Mfe-1−/− mouse livers at the age of 3–3.5 months. In our electron microscopic analysis, the space of Disse between liver sinusoidal endothelial cells and hepatocytes was affected in Amacr−/− (enlarged) and Amacr−/−Mfe-1−/− (disrupted) mice. Mfe-1−/− mice had dilated bile canaliculi, which was not so obvious in Amacr−/−Mfe-1−/− mice. The elevated liver enzyme concentrations indicated cellular damage in livers, which supports the histologic findings. Despite accumulation of potentially toxic C27 bile acid intermediates, the life spans of Amacr−/− and Amacr−/−Mfe-1−/− mice were equal to those of wild type mice, at least up to 2 years of age. However, during aging both genotypes were prone to liver tumors likely caused by the long-term accumulation of toxic C27 bile acid intermediates. The pathological analysis revealed hepatocellular carcinoma or hepatocholangiocarcinoma, oval cell hyperplasia and cholangitis in livers of the double knockout mice.

In conclusion, the results obtained in this study show that MFE-1 can contribute to the synthesis of mature bile acids via both Amacr dependent and Amacr independent pathways, thus assigning a major role for Amacr other than the indispensable role in bile acid synthesis (Figure 7). Alternative bile acid synthesis routes are consistent with the importance of bile acids and their signaling in multiple metabolic pathways.

6.3 Phytol is lethal for Amacr-deficient mice

The results of this study show that the major role of Amacr is in metabolism of α-methyl branched chain fatty acids.

Long chain α-methyl branched chain fatty acids originate from phytol, the side chain of chlorophyll that is liberated by bacteria in the stomach of ruminants. In humans phytol or its derivative phytanic acid is solely derived from nutritional sources like ruminant fats and dairy products. In healthy individuals, phytol is converted to phytanic acid, α-oxidized into pristanic acid and subsequently degraded by peroxisomal β-oxidation. Patients with AMACR-deficiency accumulate pristanic acid and to a lesser extent phytanic acid, the metabolites with demonstrated toxicity in Refsum disease (Eldjarn et al. 1966). Accumulation of these metabolites is also thought to be behind the clinical symptoms such as sensory motor neuropathy, seizures, and relapsing encephalopathy in AMACR patients (Smith et al. 2010). In mice, Amacr-deficiency results in no pathological
symptoms on a standard laboratory diet low in α-methyl branched chain fatty acids. However, a previous study showed that liver disease resulted when the diet was supplemented with phytol for six weeks (Savolainen et al. 2004).

The life span of Amacr−/− mice is comparable to wild type controls on a standard laboratory diet (Figure 7 in II). In this study mice were kept on a long-term phytol enriched diet after maturation until development of a disease state. The first Amacr−/− mouse became moribund already after two weeks on the phytol diet, and within 36 weeks all of the Amacr−/− mice became sick and were sacrificed, whereas wild type mice on the phytol diet thrived. The mortality pattern (Figure 2A in III) showed that Amacr−/− male mice did not tolerate phytol as well as Amacr−/− female mice did. However, the histological findings in liver were more prominent in Amacr−/− female mice than in male mice. This is in line with an earlier study showing that wild type C57BL/6 female mice are more susceptible to phytol (Atshaves et al. 2004). An explanation for the different mortality rates between the sexes in Amacr−/− mice could be varying expression levels of genes involved in toxic responses between the sexes. Due to the small number of Amacr−/− male mice in the survival study, conclusions are difficult to draw.

Liver dysfunction was the prominent finding in Amacr−/− mice after phytol administration. Histological analysis of liver showed inflammation, fibrotic and necrotic changes, Kupffer cell hyperplasia, and fatty vacuolization. The microscopic findings were supported by increased levels of liver marker enzymes ALAT, ASAT, and APHOS accompanied with hyperbilirubinemia in the serum. Histopathological alterations at the end point were more evident in female mice than in males, which could be attributed to their longer survival on the phytol diet. Fulminant liver failure at the end state of phytol induced disease in Amacr−/− mice might be the cause for the secondary lesions in kidney accompanied by high creatinine levels and electrolyte alterations in serum. Liver and kidney failure can be the reason for the hypoglycemia seen in Amacr−/− mice.

The phytol diet for two weeks in this study resulted in a 60-fold elevation of phytanic and pristanic acid in the liver tissue of Amacr−/− mice. An earlier study where mice were on a phytol diet for six weeks resulted in about a nine-fold elevation of phytanic acid levels and about 12-fold elevation of pristanic acid levels in the serum of Amacr−/− mice (Savolainen et al. 2004). The significant accumulation of phytanic acid in these mice on a phytol diet is somewhat unexpected since Amacr is not needed in the α-oxidation pathway. Potentially this is due to a negative feedback loop in which accumulating pristanic acid inhibits
the α-oxidation of phytanic acid. Phytanic acid accumulation is found also in the liver of Mfe-2−/− and SCP-X−/− mice after phytol feeding (Atshaves et al. 2007, Baes et al. 2000). AMACR-deficient patients also have elevated levels of phytanic acid in their serum (Ferdinandusse et al. 2000a).

Lesions seen in brain might also be secondary to liver failure although the accumulation of branched chain fatty acids and their affect on the nervous system are as yet speculative. For example, in X-ALD the central nervous system build-up of VLCFA-lipids eventually destroys the myelin sheath that surrounds the nerves, causing neurologic problems (Kemp et al. 2012, Wanders et al. 2010). VLCFAs also accumulate in the nervous system after loss of MFE-2 but the absence of a clinical phenotype despite elevated C26:0 levels in the cerebellum in Cnp-Mfp2−/− mice, a mouse model lacking MFE-2 in oligodendrocytes, does not support a crucial role for VLCFA in cerebellar degeneration in Mfe-2−/− mice (Verheijden et al. 2013). In Mfe-2−/− mice phytanic or pristanic acids do not accumulate in the cerebellum and a diet supplemented with phytol does not increase the ataxic behavior of Mfe-2−/− mice (Huyghe et al. 2006). In the Refsum mouse model phytanic acid accumulated in the cerebellum, resulted in Purkinje cell loss and phytol induced gait disturbances. Phytanic acid restriction and improvement of ataxia in Refsum patients and dosage-dependent severity of Purkinje cell degeneration in phytol fed Phyh−/− mice strongly suggest a harmful role of phytanic acid levels on the cerebellum (Ferdinandusse et al. 2008). Amacr−/− mice in this study did not display clear neurological symptoms at least in hanging and grid tests, but they did have problems in moving at the end-point of the study, although it may be due to the very sick overall status. A gait analysis would have been a better way to study motor coordination but it was not available. The lipid analysis from brain tissue of Amacr−/− mice might give further information behind the demyelination and astroglia activation seen on the phytol diet. Unfortunately the lipid analysis from brain tissues was not performed in this study.

On a molecular level, the results from microarray analysis support the findings in liver. The expression of genes involved in cholesterol biosynthesis was detected to be one of the most affected Gene Ontology pathways when comparing liver samples from wild type and Amacr−/− mice on the control diet. Administration of phytol to wild type mice caused changes in the expression of many genes, mostly related to lipid and glucose metabolism, various response reactions and the circadian clock. Because wild type mice survived easily on a phytol-enriched diet, these responses and activation of many pathways are needed
to process the toxic effects of phytol in mouse liver. Activation of pathways related to DNA segregation and cell division detected in hepatic samples from Amacr<sup>−/−</sup> mice on the phytol diet were represented as oval cell hyperplasia and bile duct proliferation in microscopic analysis. These findings indicate the regenerative capability of the liver to repair the damage caused by phytanic and pristanic acid accumulation. Pathways involved in the immune response were also observed in microarray analysis when Amacr<sup>−/−</sup> mice were fed with phytol, which supports the histological data. The mechanism behind inflammation and fibrosis resembles the pathogenesis behind non-alcoholic fatty liver disease (NAFLD) where Kupffer cells fail to eliminate toxic molecules or halt inflammation (Baffy 2009). Overall, phytol feeding to wild type and Amacr<sup>−/−</sup> mice activates different metabolic pathways reflecting also different histological status.

Accumulation of phytic acid and/or pristanic acid is a typical biochemical finding in patients with peroxisomal biogenesis disorders or single enzyme defects in peroxisomal α- or β-oxidation (Van Veldhoven 2010). Various knockout mouse models of these diseases, such as Phyh<sup>−/−</sup>, Mfe-2<sup>−/−</sup>, Scpx/Scp2<sup>−/−</sup> and Scpx<sup>−/−</sup> mouse lines (Atshaves et al. 2007, Baes et al. 2000, Ferdinandusse et al. 2008, Seedorf et al. 1998), can tolerate a phytol enriched diet for variable periods of time, but after phytol exposure hepatic changes are detected in all these mouse lines. In many cases hepatic lipid vacuoles are seen, usually accompanied by inflammatory findings. The vacuoles are likely due to accumulation of phytic and/or pristanic acid, as in the Amacr<sup>−/−</sup> mouse line. In the neonatal form of AMACR-deficiency, necrotic hepatocytes are seen, which are similar to those in Amacr<sup>−/−</sup> mice after phytol feeding (Setchell et al. 2003). Neurological findings, which are the most prominent findings in patients, are more variable in these models. Problems with male fertility were also detected in Phyh<sup>−/−</sup> and Tysnd1<sup>−/−</sup> mouse models (Ferdinandusse et al. 2008, Mizuno et al. 2013).

The results of this study show that when Amacr<sup>−/−</sup> mice are fed with phytol, the precursor of phytic acid, the lifespan is decreased. The obvious cause of death is hepatic failure aggravated by renal dysfunction. These data support the idea that the major role of Amacr is metabolism of α-methyl branched chain fatty acids derived from nutritional sources whereas cholesterol and bile acid metabolism can be readjusted to allow a normal life span on a diet very low in α-methyl branched chain fatty acids (Figure 7).
Fig. 7. The role of Amacr in degradation of branched chain fatty acids and in bile acid synthesis.
7 Conclusions

In human, \(\alpha\)-branched chain fatty acids derived from phytol are exclusively obtained from dietary sources. AMACR-deficient patients accumulate R-isomers of pristanic and phytanic acids and DHCA/THCA bile acid intermediates, and the levels of mature bile acids are reduced. The deficiency is described in early-onset subjects with bile acid deficiency with liver pathologies. The late-onset form of this deficiency is characterized by neurological disturbances. The Amacr-deficient mice generated previously unexpectedly show no clinical symptoms under standard laboratory conditions. Supplementing the diet with phytol induces a disease state in these mice.

The results of this study show that the primary physiological function of Amacr is the detoxification of \(\alpha\)-methyl branched chain fatty acids. The life span of Amacr\(^{-/-}\) mice on standard laboratory chow with low amounts of \(\alpha\)-methyl branched chain fatty acids is similar to wild type mice. Adding phytol to the diet results in the accumulation of phytanic and pristanic acids in the serum of Amacr\(^{-/-}\) mice and induces liver disease accompanied with kidney and brain lesions. Dietary phytol was shown to be lethally toxic for all Amacr\(^{-/-}\) mice within 36 weeks while wild type mice on the phytol diet thrived. The findings of this study indicate that strict dietary intervention, avoiding phytol and its metabolites, might restrain the emergence of pathologic symptoms in patients suffering from AMACR-deficiency.

The secondary role of Amacr is in the bile acid synthesis pathway, which can be compensated by alternative pathways when Amacr activity is missing. A mouse line deficient in both Amacr and Mfe-1 genes was produced in this study. Bile acid analysis from the Mfe-1 single knockout mouse line showed a reduced total bile acid pool size compared to wild type mice. The residual C24 bile acid pool size measured in Amacr\(^{-/-}\) mice was decreased further in the Amacr\(^{-/-}\) Mfe-1\(^{-/-}\) double knockout mice. Results obtained in this study using wild type, Mfe-1\(^{-/-}\), Amacr\(^{-/-}\) and Amacr\(^{-/-}\) Mfe-1\(^{-/-}\) mouse models show that Mfe-1 participates in bile acid synthesis both in Amacr independent and Amacr dependent ways. This finding provides an additional function for Mfe-1. This study shows that even though the breakdown of cholesterol in the \(\beta\)-oxidation cycle via Amacr and Mfe-2 is the main pathway, there are alternative minor pathways to break down cholesterol to C24 bile acids. The function of Amacr in bile acid synthesis can be partially circumvented assuring generation of mature bile acids that together with C27 bile acid synthesis intermediates allows the individual to survive.
How Amacr−/− mice adapt their cholesterol and bile acid metabolism to have a normal life span with a minimal amount of mature bile acids was also investigated in this study. Intestinal cholesterol absorption in Amacr−/− mice is decreased resulting in a doubling of the daily cholesterol excretion compared to wild type mice. Also fecal excretion of bile acid intermediates is enhanced 3-fold. However, the body cholesterol pool remains stable, because the Amacr-deficiency speeds up hepatic sterol synthesis 5-fold. Changes in lipoprotein profiles seen in Amacr−/− mice are mainly due to decreased phospholipid transfer protein activity. At the molecular level, these adaptations can be manifested as changes in hepatic and intestinal metabolism through nuclear receptor transcription factors. Concentrations of the ligands of these factors are apparently modified. In conclusion, life with a minimal amount of mature C24 bile acids is possible, but it is a matter of the environment where one lives, and whether the diet contains branched chain fatty acids that require Amacr to degrade them.

The mechanism of how phytol supplementation induces demyelination in the brains of Amacr−/− mice remains to be elucidated. Also the physiology of adaptation, how changes in the lipoprotein profile develop, requires further investigation. The intervention test where Amacr−/− mice are administered mature bile acids will most likely correct the cholesterol metabolism, but will not improve the life span of Amacr−/− mice on a phytol diet and will not repair the detoxification of phytol metabolites. In the future we should also look into the role of Amacr in mitochondria. Amacr is overexpressed in various cancers but the exact mechanism for this is unresolved and is under vigorous investigation throughout the world.
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