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STUDIES ON THE OXYSTEROL RECEPTOR LXRβ: LINKING CHOLESTEROL METABOLISM TO WATER TRANSPORT AND CELL PROLIFERATION

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“We can not all do great things,
but we can do small things with great love”.
(Mother Teresa)

To my mother.

ABSTRACT

Liver X Receptor β (LXRβ) is a nuclear receptor, belonging to the superfamily of ligand-activated transcription factors. With its α isoform (LXRα), LXRβ shares more than 78% homology in its amino acid sequence, a common profile of oxysterol ligands and the heterodimerization partner, Retinoid X Receptor. LXRs have a crucial role in lipid metabolism, in particular in preventing cholesterol accumulation, in glucose homeostasis and in macrophage inflammatory response. The first evidence that, in spite of all the common properties, LXRα and LXRβ have distinct functions, came in 2001 with the creation of knock-out mice for each LXR isoform. LXRα-/- mice fed with 2% cholesterol diet show a severe cholesterol accumulation in the liver due to an inability to increase bile acid synthesis in response to high cholesterol intake. Surprisingly, LXRβ-/- mice had the same compensatory capacity of WT mice to avoid hepatic cholesterol accumulation suggesting that LXRβ may have a completely distinct role from LXRα. Indeed in 2005, it was shown that specifically in LXRβ-/- male mice cholesterol accumulates in big motor neurons of the spinal cord leading to their death and inducing a significant motor function impairment like amyotrophic lateral sclerosis (ALS). Starting from this neurological phenotype of LXRβ-/- mice and comparing the characteristics of knock-out mice for each LXR isoform, this thesis aims to define new and specific functions of the oxysterol receptor LXRβ.

Paper I of this thesis aims to investigate the neurological phenotype of LXRβ-/- mice focusing in particular on the role of β-sitosterol in the pathogenesis ALS-Parkinson-Dementia complex. Administration of β-sitosterol to LXRβ-/- mice creates a severe motor-impairment and loss of dopaminergic neurons in the substantia nigra, activates microglia and decreases brain cholesterol indicating that LXRβ may have a protective role against the toxic action of β-sitosterol on the central nervous system.

Paper II investigates the resistance to gain weight, characteristic of LXRβ-/- mice and demonstrates that they are affected by a severe pancreatic insufficiency with low serum levels of amylase, lipase, low fecal protease and abundant inflammatory infiltrates all around medium size pancreatic ducts. The water channel aquaporin-1 (AQP-1), responsible of transporting water into the pancreatic ductal lumen was markedly decreased in LXRβ-/- mice leading to the presence of plugs inside the ducts and in turn to a pancreatic insufficiency.

In the digestive system AQP-1 is strongly expressed in the cholangiocytes of the gallbladder being, together with AQP-8, the mediator of the absorbing-secretory functions of this organ. **Paper III** shows that the male gallbladder cholangiocytes of LXRβ-/- mice express very low mRNA and protein levels of both AQP-1 and AQP-8 and morphologically they appear shrunk with loss of cell polarization. Treatment of WT mice with LXR-agonist increases the expression of the two water channels in the gallbladder together with the cholesterol transporters ATP Binding Cassette G5/G8 and it is associated with cholesterol crystals in the bile.

The morphology of female LXRβ-/- gallbladders was studied in **paper IV**: at the age of 12 months a wide range of preneoplastic lesions are detectable, from dysplasia to metaplasia and adenomas, degenerating into carcinoma in situ, when the mice become 19 months old. The pathogenesis involves a complex interplay between LXRβ, Transforming Growth Factor β (TGFβ) and estrogens. Indeed, ovariectomy of LXRβ-/- mice prevents the development of preneoplastic lesions and normalizes the TGFβ signaling that is upregulated in LXRβ-/ mice.

In conclusion, this thesis describes new emerging and specific roles for LXRβ in controlling not only cholesterol homeostasis in the central nervous system but also water channels in pancreas and gallbladder as well neoplastic transformation of cholangiocytes.

LIST OF PUBLICATIONS

I. Kim HJ, Fan X, **Gabbi C**, Yakimchuk K, Parini P, Warner M, Gustafsson JA. Liver X receptor β (LXRβ): a link between β-sitosterol and amyotrophic lateral sclerosis-Parkinson's dementia. Proc Natl Acad Sci 2008;105:2094-9

II. **Gabbi C**, Kim HJ, Hultenby K, Bouton D, Toresson G, Warner M, Gustafsson JÅ. Pancreatic exocrine insufficiency in LXRβ-/- mice is due to a reduction in aquaporin-1 expression. Proc Natl Acad Sci, 2008; Sep 30;105(39):15052-7

III. **Gabbi C**, Kim HJ, Hultenby K, Warner M, Gustafsson JÅ. LXRβ, the physiological regulator of the expression of aquaporin-1 and aquaporin-8 in gallbladder cholangiocytes. Manuscript

IV. **Gabbi C**, Kim HJ, Barros R, Korch-Andre M, Warner M, Gustafsson JÅ. Estrogen dependent gallbladder carcinogenesis in LXRβ-/- mice. Proc Natl Acad Sci 2010; 107(33):14763-8

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LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette
ACC	Acetyl-CoA Carboxylase
AF	Activation Function
ALS	Amyotrophic Lateral Sclerosis
ALS-PDC	Amyotrophic Lateral Sclerosis -Parkinson Demetia Complex
ApoE	Apolipoprotein E
AQP	Aquaporin
COX-2	Cyclooxygenase-2
ChREBP	Carboydrate Responsive Element Binding Protein
CSF	Cerebro Spinal Fluid
CYP27A1	Sterol 27-hydroxylase
CYP46A1	Cholesterol 24-hydroxylase
CYP7A1	Cholesterol 7alpha hydroxylase
CYP7B1	Oxysterol 7alpha-hydroxylase
DBD	DNA Binding Domain
DR4	Direct Repeat 4
ELISA	Enzyme-linked immunosorbent Assay
ER	Estrogen Receptor
FAS	Fatty Acid Synthase
FFA	Free Fatty Acids
FXR	Farnesoid X Receptor
G-CSF	Granulocyte colony-stimulating Factor
GABA	γ-Aminobutyric acid
GLUT-4	Glucose transporter type 4
GR	Glucocorticoid Receptor
HC	Hydroxy Cholesterol
HDL	High Density Lipoprotein
iEM	Immuno Electron Microscopy
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
LBD	Ligand Binding Domain
LPS	Lipopolysaccharides
LXR	Liver X Receptor
LXRE	LXR Responsive Element
MCP	Monocyte Chemotactic Protein
MIP	Macrophage Inflammatory Proteins
MMP-9	Matrix Metallopeptidase 9
NCoR	Nuclear Receptor Coactivator
NF-kB	Nuclear Factor kappa B
NR	Nuclear Receptors
PCNA	Proliferating Cell Nuclear Antigen
PGC1-α	Peroxisome Proliferator Activated Receptor γ coactivator 1-α
PPARγ	Peroxisome Proliferator Activated Receptor γ
PPI	Proton Pump Inhibitor
PUFA	Polyunsaturated Fatty Acids
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RXR	Retinoid X Receptor
SCD-1	Stearoyl-CoA Desaturase-1
SHP	Small Heterodimer Partner
SREBP-1c	Sterol Regulatory Element Binding Protein
SULT2A1	Sulfotransferase 2A1
SUMO	Small Ubiquitin-like Modifier
TEM	Transmission Electron Microscopy
TGFβ	Transforming Growth Factor β
TLR	Toll Like Receptor
TNFα	Tumor necrosis factor α
TUNEL	Terminal Transferase dUTP Nick End Labeling

1 INTRODUCTION

1.1 NUCLEAR RECEPTORS

1.1.1 Milestones in nuclear receptor research

Nuclear Receptors (NR) are a large super family of transcription factors whose discovery has opened new frontiers in understanding not only the endocrine action of steroid hormones but also and especially, the “hormonal behavior” of canonical non-hormonal molecules such as oxysterols, bile acids and vitamins.

Some members of this superfamily are ligand-activated, and act as transcription factors upon binding to small biologically active molecules. Activated receptors can bind to specific DNA sequences (response elements) in the promoter of target genes, or can interact with other transcription factors to activate or inhibit transcription.

The epoch of nuclear receptor research started at the end of 1950s with the observation that the injection of radioactive estradiol into rats had a tissue specific uptake and retention pattern, indicating the existence of a protein capable of binding to estradiol [1-3]. These studies by Elwood Jensen culminated in the identification in the uterus of estrophilin, an estradiol-binding protein, afterwards named estrogen receptor (ER) and subsequently identified as a nuclear receptor [4].

A plethora of subsequent studies, in particular the action of progesterone on chick oviducts, led to our present understanding of the physiological steps in nuclear receptor signalling. It is generally accepted that steroid hormones bind to their specific receptors which are located either in the nucleus or cytoplasm. Cytoplasmic receptors migrate to the nucleus upon binding to their ligands. In the nucleus, activated receptors bind to specific sites on DNA and induce the transcription of specific mRNA and in turn the synthesis of protein involved in tissue differentiation, proliferation or metabolism [5-8].

It was at the end of 1970s that the first nuclear receptor, the glucocorticoid receptor (GR) was purified [9] and its three domains were identified: a ligand-binding domain (LBD), a DNA-

binding domain (DBD) and a third strongly immunogenic domain [10]. Thanks to the highly conserved structure of nuclear receptors and their homology in the DBD, in “the cloning era” of the 1980s, it was possible to clone many previously unknown nuclear receptors. Thus in a process called “reverse endocrinology”, it was discovered that there were many more nuclear receptors than there were steroid hormones. Those receptors whose ligands were not known were called “orphans” [11].

Over the past 15 years, 48 members of the NR superfamily have been identified in the human genome and many NR ligands are now targets for pharmacological interventions [12].

1.1.2 Structure

Nuclear receptors share a canonical structure, composed of functionally distinct domains (Figure 1): the N-terminal activation function 1 (AF1) domain, highly variable in sequence and length [13, 14]; the highly conserved DNA-binding domain (DBD) that contains two zinc-binding motifs, involved not only in DNA binding but also in receptor dimerization [15]; and the C-terminal ligand binding domain (LBD) with a key role in ligand binding, nuclear localization, receptor dimerization and interaction with coactivators and corepressors [16, 17]. Between the DBD and LBD is the hinge domain that provides flexibility between these two domains. The AF2 domain lies within the LBD. AF2 adopts different conformations depending on the structure of the ligand which is bound in the ligand-binding pocket. In general, agonists induce conformations that are recognized by coactivators, while antagonists induce conformations recognized by corepressors.

1.2 LIVER X RECEPTORS

Liver X Receptors (LXRs) are nuclear receptors first identified as “orphans” but subsequently adopted by oxygenated cholesterol derivates [18]. There are two isoforms with 78% amino acid homology in their DNA-binding domain and ligand-binding domain. LXRα (NR1H3), first discovered by Magnus Pfahl and called RLD1 [19, 20], and LXRβ (NR1H2) [21] also named

ubiquitous receptor [22], NER [23] or orphan receptor-1 [24] because of its concomitant independent discovery by four different laboratories. In humans, LXRα is located on chromosome 11p11.2 and LXRβ on chromosome 19q13.3.

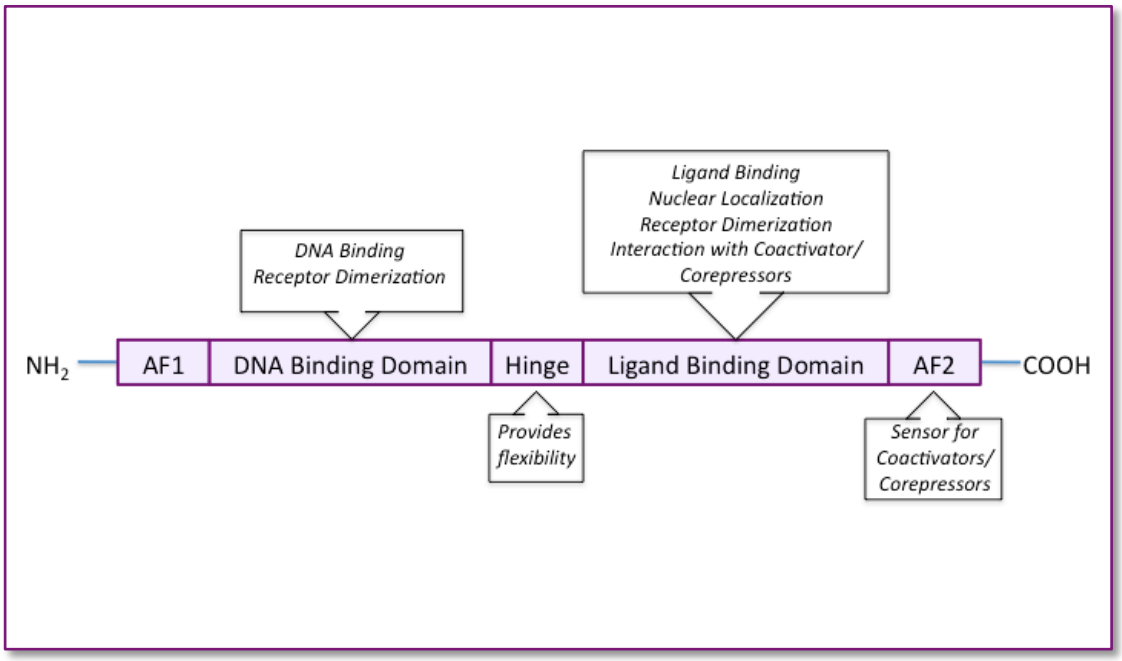

Figure 1. Conserved structure of Nuclear Receptors containing the following domains: the N-terminal activation function 1 (AF1) domain, highly variable between nuclear receptors; the DNA-binding domain very conserved between members; the hinge region, a flexible domain between the DBD and LBD; the ligand-binding domain involved in the interaction with ligands; the AF2 domain that is a part of LBD whose different conformations are dictated by the type of ligand bound and are recognized by coactivators and corepressors.

1.2.1 Tissue distribution of LXRs

In adult mice, mRNA of the two LXRs isoforms have been detected with a different distribution profile. LXRα is highly expressed in the liver, adipose tissue, intestine, kidney, and macrophages while LXRβ mRNA is ubiquitously expressed with high levels in the developing brain [25, 26].

During mouse development, starting from embryonic day 11.5, both LXRα and LXRβ mRNA are detected in the liver. LXRα maintains high expression throughout life while, hepatic LXRβ decreases during later embryonic development [27]. Between mouse embryo ages days 11.5 and 16.5, LXRα mRNA appears to be detectable in brown adipose tissue, thyroid gland, and intestine while LXRβ mRNA is strongly expressed in brain, retina, ganglia (tibulocochlear, trigeminal, dorsal root), kidney, adrenal, thymus and thyroid gland [27].

In the brain, LXRβ protein expression is detectable as early as embryo age day 14.5 in the neurons of the cortical plate [28].

1.2.2 Ligands

A wide range of molecules, both natural and synthetic has been shown to be potential ligands of LXR in *in vitro* assays [29].

The first identified natural ligands that can activate LXRs at physiological concentration are oxysterols, in particular 24(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, 24(S),25-epoxycholesterol, 27-hydroxycholesterol [18] and its metabolite, cholestenoic acid [30]. The synthesis of 24(S)-hydroxycholesterol from cholesterol is catalysed by the enzyme cytochrome P450 46 A1 (CYP46A1). This is a key pathway in brain cholesterol homeostasis since it is the main mechanism of cholesterol removal from the brain [31]. 22(R)-hydroxycholesterol is a naturally occurring oxysterol while 24(S),25-epoxycholesterol is made in a shunt during cholesterol synthesis pathway from the cholesterol precursor squalene [32]. 27-Hydroxycholesterol is generated by a mitochondrial P450 enzyme, CYP27, involved in the alternative bile acid synthesis pathway [33].

It is therefore intriguing that both enzymes metabolizing and catabolizing oxysterols, respectively, may participate in the regulation of LXR activity. Emerging in vivo studies support this notion. Knockout mice engineered to delete enzymes synthesizing 24(S)-HC, 25-HC and 27-HC are unable to induce LXR target genes in response to dietary cholesterol but remain responsive to a synthetic LXR agonist (T0901317) [34]. Moreover, treatment of mice with inhibitors of cholesterol synthesis such as the archetypal statin, compactin, leads to a decrease in the synthesis of 24(S),25-epoxycholesterol, and to a decreased expression of LXR target genes [35, 36]. Conversely, in mice, adenovirus-mediated overexpression of cholesterol sulfotransferase, (SULT2A1) an enzyme capable of catabolizing oxysterols, prevents dietary induction of hepatic LXR target genes by dietary cholesterol but not by T0901317 [34].

D-glucose has been reported capable of binding to both LXRα and LXRβ and inducing LXR transcriptional activity [37]. This role of LXRs as glucose sensors is not well understood since only the transcription factor carbohydrate-responsive element binding protein (ChREBP), and not LXRs, has been shown to induce glucose-regulated genes in the liver in presence of glucose [38].

Phytosterols, in particular β-sitosterol have also been recognized as ligands for LXRs [39].

Moreover, two non-steroidal synthetic compounds, GW3965 and T0901317 have been identified as LXR agonists [40, 41] capable of activating both LXRs isoforms. T0901317 is less specific than previously thought since it can also activate the bile acid receptor, Farnesoid X Receptor (FXR) even more potently than its natural ligand chenodeoxycholic acid [42] and it may act as an activator even of the Pregnane X Receptor (PXR) at the same concentrations at which it activates LXRs [43]. Therefore at present GW3965 appears to be the most selective synthetic LXR ligand.

Recently, members of the Proton Pump Inhibitor (PPI) family, such as lansoprazole, pantoprazole and omeprazole, have been described as LXR activators in several cell culture systems including primary mouse glial cells. The stimulatory effect of these PPIs on LXR transcription of LXR-regulated genes was abolished in LXRα-/-β-/- glial cells [44].

In terms of selective ligands, a subset of natural bile acids has been reported to activate LXRα [30] whereas N-acylthiadiazolines have selectivity for LXRβ but with modest potency [45].

1.2.3 Mechanism of action

LXRs have been shown to regulate gene transcription through two different mechanisms of action: direct activation and transrepression (Figure 2).

1.2.3.1 Direct gene activation

LXRs form obligate heterodimers with the Retinoid X Receptor (RXR) [19] and bind to specific nucleotide sequences called LXR-responsive elements (LXREs) consisting of a direct repeat of the core sequence 5'-AGGTCA-3' separated by 4 nucleotides (DR4) [46] in DNA of target genes. Inverted repeat of the same sequence with no space region (IR-0) or with 1 bp spacer (IR-1) have also been shown to mediate LXR transactivation [47, 48].

In the absence of ligands, LXRs are in a non-active state, binding to cognate LXREs in complex with corepressors such as the Nuclear Receptor Corepressor (NCoR) or the Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor (SMRT) [49, 50]. The binding of ligands induces a change in the conformation of LXRs that enables the release of corepressors, recruitment of coactivators [51] and in turn the direct activation of gene transcription. Several coactivators have been described for LXRs . These include: Peroxisome Proliferator Activator Receptor-γ (PPARγ) coativator-1α (PGC-1α) [52], the Steroid Receptor Coactivator-1 (SRC-1) [53] and the Activating Signal Cointegrator-2 (ASC-2) [54].

1.2.3.2 Transrepression

Due to transrepression, LXRs, in particular LXRβ [55] exert a strong inhibition on the transcription of NF-kB regulated proinflammatory genes [56] that lack a direct binding site for LXRs. After binding of the ligand, LXRβ undergoes a specific SUMOylation by SUMO-2/3 that promotes interaction with GPS2, a subunit of the N-CoR complex. In this setting the

dissociation of the N-CoR complex from NF-*k*B is prevented and in turn the transcription of pro-inflammatory genes is blocked [55].

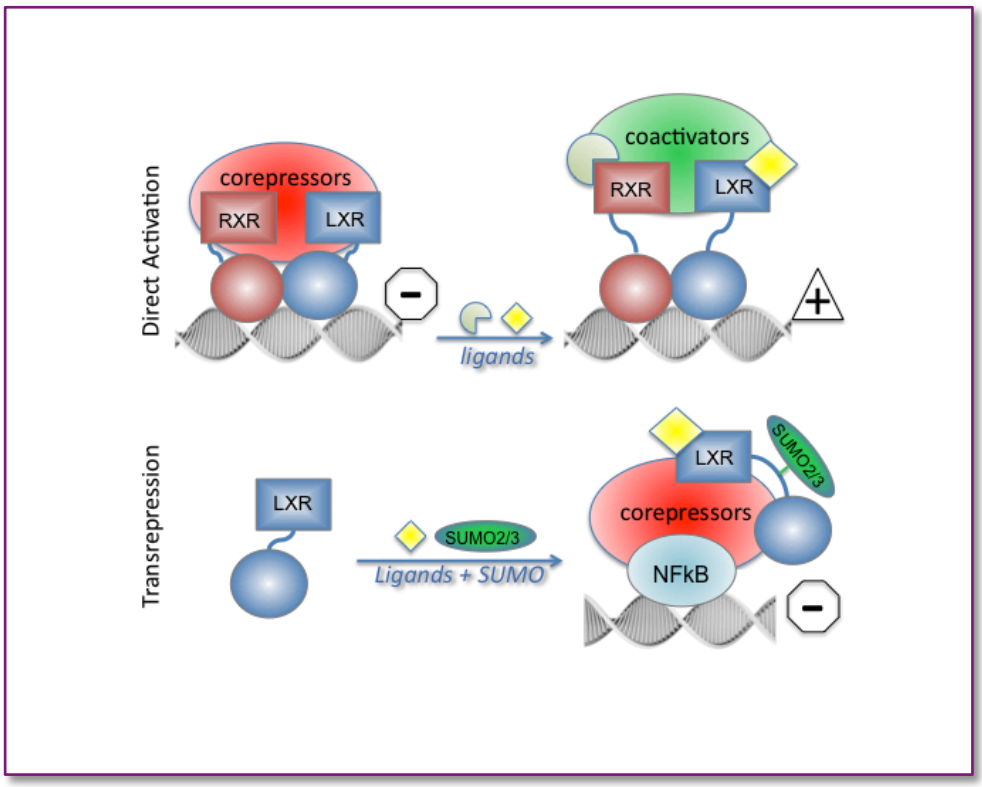

Figure 2. *LXR*s influence gene expression by (i) directly promoting gene transcription after heterodimerization with RXR, binding with the ligands and interaction with coactivators and (ii) by transrepressing NF-kB regulated genes after SUMOylation and interaction with corepressors.

1.2.4 Nuclear Receptors influencing LXR activity

As described, LXR transcriptional activities are the result of a complex balance between bioavailability of ligands and their related metabolizing/catabolizing enzymes, the presence of coactivators and corepressors, the SUMOylation process and even the influence of other nuclear receptors such as PPARγ and Small Hetherodimer Partner (SHP).

Indeed PPARγ, a nuclear receptor activated by fatty acids as well as their oxidized metabolites, has been shown to induce the expression of LXRα in macrophages [57]. Furthermore, SHP, a direct target gene of FXR, is capable of interacting with LXRα and blocking its transcriptional activity [58]. In the liver, SHP is one of the main effectors of the negative feedback regulation on CYP7A1, the rate limiting enzyme in the bile acid synthesis pathway [59].

Moreover, in adipose tissue, LXRα transcriptional activity appears to be estrogen-regulated and, in the LXRα promoter, a sequence that is negatively regulated by estrogens has been identified [60].

1.2.5 LXR in metabolic control

1.2.5.1 Cholesterol homeostasis

LXRs act as “sterol sensors”: oxysterols activate LXRs and thus increase transcription of genes involved in cholesterol catabolism and excretion.

In the liver, LXR activation promotes cholesterol elimination by inducing the expression of CYP7A1, the rate limiting enzyme in the classical pathway of bile acid biosynthesis [61], as well as the expression of the ATP-binding cassette transporters, ABCG5/G8 that transport cholesterol from the hepatocytes into the bile canaliculi [62]. Indeed LXRα-/- mice fed a normal diet have normal hepatic cholesterol levels [51, 63, 64] but a decreased bile acid pool. Administration of 2% cholesterol diet to LXRα-/- mice clearly shows their inability to eliminate cholesterol by its conversion to bile acids with a consequent accumulation of cholesterol esters in the liver [51, 64]. Surprisingly, on the same diet, LXRβ-/- mice have a similar response

as WT mice: hepatic cholesterol levels are normal as well as the expression of the enzymes involved in bile acid metabolism (CYP7A1, CYP7B, CYP8B1, CYP27) [63] indicating that of the two LXRs it is LXRα which controls liver cholesterol balance. LXRs also protects extrahepatic tissues from cholesterol accumulation. The main mechanism of this protection is through the control of cholesterol reverse transport. In macrophages, LXR agonists induce the expression of ABCA1, ABCG1 and ABCG4 transporters that promote the efflux of cholesterol to high density lipoproteins (HDL) [65, 66]. The observed accumulation of foam cells rich in cholesterol esters, in the aorta, spleen, and lung of LXRα-/-β-/- mice is thought to be a result of an impaired reverse cholesterol transport [64].

1.2.5.2 Fatty acid metabolism

The study of LXR knock-out mice also gave important insights into LXR physiology and the role of LXRs in fatty acid metabolism. Indeed liver triglycerides are significantly reduced in LXRα-/-β-/- mice [63] and treatment with LXR-agonist leads to development of hepatic steatosis in WT mice [41] as a consequence of an upregulation of genes involved in fatty acid synthesis (scd1, fas, srebp-1c).

1.2.5.3 Glucose homeostasis

A role for LXRs in controlling glucose homeostasis has been demonstrated in several animal models. Treatment of diabetic mice and Zucher rats with an LXR agonist is associated with improvement of glucose tolerance and decrease in gluconeogenesis [67, 68]. In parallel, in adipose tissue, LXRs positively control the expression of the insulin-dependent glucose transporter 4 (GLUT4) [69] that mediates the uptake of glucose from peripheral blood.

1.2.6 LXRs in inflammatory response

Besides the previously described metabolic actions, there is emerging evidence that LXR, in particular LXRβ [55], may act as key effectors in the integration between lipid and inflammatory signals, both in vitro and in vivo.

In peritoneal and bone marrow-derived macrophages, pre-treated with LXR agonists, there is reduced expression of inflammatory genes in response to bacterial pathogens as well as to stimulation with LPS, TNFα, or IL-1β. This effect was paralleled with the induction of cholesterol transporters, like ABCA1 and was abolished in macrophages lacking both LXR isoforms [70, 71]. The profile of LXR-anti-inflammatory action is defined by reduced inducible Nitric Oxide Synthase (iNOS) mRNA, protein and activity; inhibition of COX-2 protein expression; suppression of numerous genes involved in macrophage innate immune response such as the cytokines IL-6, IL-1β, the granulocyte-colony stimulating factor (G-CSF), the chemokines MCP-1, MCP-3 (Monocyte Chemoattractant Protein), the Macrophage Inflammatory protein-1β (MIP-1β) [71] and the metalloproteinase MMP-9 [70, 72].

In an anti-inflammatory setting, LXRs may also directly upregulate the expression of *Arginase-II* gene (*ArgII*) in macrophages [73]. ArgII catalyses the conversion of L-arginine to L-ornithine, therefore competing with iNOS for the common substrate arginine [74] leading to reduced production of cytotoxic NO and therefore reduced inflammatory activity.

Numerous *in vivo* studies strongly support the anti-inflammatory action of LXR-agonists both in the treatment and prevention of inflammatory diseases, including atherosclerosis. LXR clearly shows a double role by regulating both metabolism and inflammation in murine models of atherosclerosis: treatment of ApoE-/- mice with an LXR agonist induces a reduction in serum total cholesterol [72] as well as in MMP-9 expression in the aorta [71] resulting in a decreased area of atherosclerotic lesions.

Other impressive effects of synthetic LXR-agonists have been described in skin where topical application of these compounds can reverse both atopic and irritant dermatitis in hairless mice [75] as well as in BL6 mice [71, 76]. In these models, edema and inflammatory infiltration

are reduced [71, 76] together with lower immunoreactivity of TNF-α and IL-1α [76]. Natural LXR ligands are also effective in ear dermatitis [76], as well as in irritant and allergic but not in atopic dermatitis of hairless mice [75]. Topical treatment with LXR-agonist has shown promising results even in the prevention of wrinkle formation in a mouse model of photoaging [77].

Strong beneficial effects of LXR agonists have also been obtained in the CNS. In murine models of spinal cord injury [78], Alzheimer's disease [79], acute encephalomyelitis [80] and global brain ischemia [81] the amount of inflammatory infiltrate, expression of cytokines as well as clinical outcome are significantly alleviated by LXR agonists.

In the respiratory system more diverse actions of LXR agonists have been observed. Oral pre-treatment with LXR agonist prevents severe inflammatory events in mice undergoing nasal instillation of LPS [82, 83] and intra-peritoneal administration of LXR agonist reduces inflammation in a carrageen-induced pleurisy [84]. On the other hand, murine models of allergy and asthma display an increased airway reactivity and bronchial smooth muscle thickness [85] from high doses of LXR agonist.

Different effects of LXR agonists are also seen in murine collagen-induced arthritis: increased articular inflammation and cartilage destruction have been described as adverse events of both GW and T0901317 given ip [86] at a dose of 10-30 mg/Kg for 6 days. However, lower doses of GW3965 (0.1-1 mg/Kg) have been shown to improve arthritis, clinically, histopathologically and in reducing pro-inflammatory cytokines [87].

These discrepancies may in part be explained by the fact that high doses of LXR agonist administered during a relatively long period of time could exert an antagonistic effect on LXR as described by ourselves [88] and others [72]. Even the route of administration, the property of the solvent, the severity of the pre-existing disease as well as sex and age of the animal, may affect the pharmacological properties of LXR agonists in vivo and, therefore, explain opposite effects of the same compound. More pharmacokinetic and pharmacodynamic studies are required for a safe anti-inflammatory use of synthetic LXR ligands.

Mechanistically, the described anti-inflammatory action of LXR is exerted through a transrepression on the activity of the pro-inflammatory transcription factor NF-*k*B [71] that has been discussed above (Figure 2) [55, 56].

Moreover, in the scenario of a crosstalk between metabolism and inflammation, it has been reported that infectious agents, like bacteria or viruses, inhibit LXR signaling by activating Toll Like Receptor 3 (TLR3) and TLR4 in cultured macrophages as well as in aortic tissue *in vivo* [70]. Even with the activation of both LXR and TLR3/4, cholesterol efflux from macrophage is markedly decreased. It appears that the interferon regulatory factor 3, IRF 3 may be the mediator of the repression of LXR activity [70].

1.2.7 LXRs in cell cycle control

Antiproliferative and pro-apoptotic effects of LXR activation have been described in a wide set of cell culture systems ranging from primary pancreatic β-cells to breast, ovarian, prostate, stomach, and liver tumor cell lines.

In pancreatic islets and β-cell cultures, where both LXRα and LXRβ are expressed with a prevalence of LXRβ [89, 90], treatment with an LXR agonist (T0901317) decreases the rate of cell proliferation in a dose-dependent manner starting from 5 μmol/l for 48 h [90]. At lower doses (1-2 μmol/l), LXR-agonist exerts the antiproliferative activity only in the presence of the RXR agonist 9-cis-retinoic acid [91]. The mechanism underlying this cell cycle arrest in Go/G1 phase following LXR activation is still not completely understood. An increase in p27 protein level has been described as a possible responsible mechanism and evidence for this is supported by the ability of p27 siRNA to prevent the effect of LXR on cellular proliferation [91]. In addition, a proapoptotic effect of LXR-agonist has been shown in pancreatic islets and β-cell cultures [90-92], together with an increased lipogenic activity (due to an activation of LXR target genes, ADD, FAS, ACC) resulting in intracellular high levels of TG and free fatty acids [92]. This observation may indicate that the pro-apoptotic effect of LXR is due to a lipotoxic damage [93]. Indeed in several cell-systems obtained from prostatic tissue (RWPE1, LNCaP),

stomach cancer (SNU16) and hepatocellular carcinoma (HepG2), LXR-agonist (T0901317 and GW3965) induces lipogenic genes (SREBP-1c, FAS), increases levels of TG and FFA and arrests the cell cycle in G0/G1 phase [93]. These effects are markedly reduced after knock-down of FAS with siRNA.

In a more complex interplay involving also androgen signaling, LXRs participate in the control of prostate cancer cell proliferation. Both synthetic (T0901317) and natural ligands (22(R)-HC and 24(S)-HC) are effective in inhibiting cell growth in particular in androgen-independent LNCaP cells [94] both *in vitro* and *in vivo*. Indeed, in athymic nude mice, LXR agonist treatment inhibited the growth of LNCaP tumor xenografts [94] and delayed the progression to androgen-independent tumors [95]. Although the mechanism of action is still unknown, it should be noted that T0901317 may act as competitive antagonist on androgen receptor [96]. Nevertheless, *in vivo* role of LXRs, in particular LXRα, in prostate pathophysiology is supported by studies in LXRα-/- mice in which the ventral prostate is affected by a smooth-muscle actin-positive stromal overgrowth [88]. Mechanistically, the transforming growth factor β (TGFβ) signaling seems to be involved since the expression of snail and smad-2/3, downstream genes of TGFβ, was markedly increased in the ventral prostate of LXRα-/- mice [88].

1.2.8 LXRs in embryogenesis

Studies from our own laboratory have demonstrated that LXRβ has an important role in the development of cerebral cortex. At late stage of embryogenesis (E 18.5) and in neonates (P2), LXRβ-/- mice have a smaller brain with a reduction in the number of neurons in the superficial cortical layers. During development, neurons migrate from lower layers to superficial layers. After birth (P2), in LXRβ-/- mice the number of neurons is higher in lower cortical layers (IV) while in WT mice more neurons are in the upper layer (II-III) indicating a migration defect [28].

1.2.9 LXRs genetics in human diseases

The role of genetic mutations or gene polymorphisms of LXRs in human diseases corresponding to the phenotypes described in the transgenic animals is relatively unexplored; at the moment only three such studies have been published.

Several single nucleotide polymorphisms (SNPs) of LXRβ (chromosome 19) have been identified: LXR1 in intron 5, LXR2 in intron 7, LXR3 in the 3′UTR and LXR4 in intron 2. An association between the risk of developing late-onset (age at onset after 60 years) Alzheimer disease and LXR2 and LXR4 has been shown in an American population of 931 Alzheimer disease patients [97]. Although LXR2 seems to be a silent SNP, LXR4 is likely to be functional, residing in either a coding region or in a splicing junction. Moreover, this association has been confirmed in a Spanish population of 414 Alzheimer disease patients. In this study there was an increased risk if these SNPs (LXR2, LXR4, LXR1) are associated with a SNP in heme-oxygenase-1 (413 TT) [98].

The third study involves a Swedish population of 559 obese patients. This study revealed that one LXRα (rs2279238) and two LXRβ SNPs (LB44732G>A and rs2695121), in the promoter region and in intron 2, are associated with obesity [99].

More studies are required to confirm that these SNPs have a functional role in the susceptibility to Alzheimer disease and obesity.

1.3 AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disorder characterized by progressive loss of motor neurons in the spinal cord, in the cortex and in the brain stem. The worldwide prevalence of ALS is 4-6 per 100,000 inhabitants with an incidence of 0.5-3 per 100,000 yearly [100]. Approximately 10% of ALS cases are familial (FALS) with a genetic autosomal dominant trait, while the remaining 90% of cases are sporadic (SALS) [101]. In familial cases, the prevalence of affected males is much higher (male:female ratio 7:1) but this gender difference is reduced by increasing age of presentation, reaching a 1:1 ratio in

patients in their eighth decade [100, 101]. Typically this disease is fatal within 3-5 years of the onset of symptoms.

Clinically, FALS and SALS are indistinguishable but a distinct manifestation associated with parkinsonism-dementia, called PD Complex (PDC) is seen in the pacific islands of Guam. In the indigenous Chamorro population of this island, the prevalence of ALS is strongly higher than elsewhere in the world with a more malignant clinical appearance [102]. The etiopathogenesis of PDC is still unknown but both genetic and environmental factors are thought to be involved. The characteristic pathological finding at autopsy is the high prevalence of neurofibrillary tangles (NFTs) in patients with PDC. Interestingly, in comparison with control American subjects, healthy Chamorros also have an increase in neurofibrillary tangles [103].

Leucine-rich repeat kinase 2 (LRRK-2), a protein mutated in familial Parkinson disease with unclear function, has been shown to accumulate in these tangles and TDP-43, a transcriptional repressor normally expressed in the nucleus, accumulates in glial inclusions [104]. One interesting hypothesis on the role of diet in the etiology of ALS involves the chronic exposure to toxins from *Cycas micronesica*. This is a palm from which the flour has traditionally been prepared and used as the major source of flour when wheat is scarce. Feeding of monkeys with up to 2 g of cycad flour does not lead to any neurological disease [105]. Thus it is thought that the indigenous Guam population has a genetic predisposition which renders them susceptible to the toxic effects of cycad flour.

Still unknown is also the pathogenesis of the pure sporadic form of ALS. In familial cases a mutation of SOD1 gene has been described [106]. SOD1 is a Cu/Zn-binding superoxide dismutase that catalyzes the dismutation of toxic superoxide anion O_2^- to O_2 and H_2O_2 [107]. The hypothesis is that in FALS patients the activity of SOD1 could be either reduced, leading to an accumulation of toxic superoxide radicals or, more probably, increased leading to excessive levels of H_2O_2 that can react with some metals like iron and generate highly toxic hydroxyl radicals [108].

1.4 MALABSORPTION SYNDROME

Malabsorption syndrome is a clinical condition characterized by a combination of symptoms like weight loss or growth failure in children, steatorrhoea, diarrhea, and anaemia which result from unsuccessful nutrient absorption from the diet. Numerous diseases are responsible for this syndrome and according to their etiology, they can be classified into three groups: (a) alterations of the digestive process due to deficit of enzymes and bile acids such as in chronic pancreatitis, cystic fibrosis, and cholestatic liver diseases; (b) alterations in uptake and transport due to a damage of absorptive surface such as in celiac disease, Crohn´s disease, and autoimmune enteropathy; (c) microbial causes such as bacterial overgrowth and parasitosis [109]. The major cause of defective intraluminal digestion is pancreatic exocrine insufficiency due to chronic pancreatitis and cystic fibrosis. In industrialized countries, the incidence of chronic pancreatitis is between 3.5-10 per 100.000 inhabitants. About 70-80 % of cases are related to long-term alcohol misuse while 10-30 % of cases represent idiopathic pancreatitis for which the etiology is still unknown [110]. A large number of mutations in genes coding for serine protease inhibitor, SPINK1, or the cystic fibrosis transmembrane conductance regulator, CFTR, have been described to be involved not only in the pathogenesis of pancreatitis but also, working in concert with other genetic and environmental factors, in the susceptibility to this disease [111]. Moreover, in humans, it has been shown that genetic polymorphisms of genes regulating the inflammatory response, like heat shock protein 70-2 or tumor necrosis factor α, are associated with an increased risk of acute pancreatitis [112].

1.5 GALLBLADDER CANCER

Carcinoma of the gallbladder is a highly fatal and aggressive disease with a poor prognosis. It is the most common malignant tumor of the biliary tract with 5000 estimated new cases per year in United States [113]. Incidence of gallbladder carcinoma varies with sex and ethnicity. Women are affected two to six times more than men and the highest incidences

are reported in Native Americans, South American populations, people from Poland and North of India [114].

The etiology of gallbladder carcinoma involves a complex interplay between hormones, metabolic alterations, infections and even anatomical anomalies [115]. Epidemiological studies have shown a strong association of this tumor (in particular the squamous and adenosquamous variant) with cholesterol gallstone disease [116] and with many of its risk factors like obesity, high carbohydrate intake and female sex [117]. The strong female incidence has raised the hypothesis that estrogens could play an important pathophysiological role in the development of gallbladder cancer. It has been shown that Hormone Replacement Therapy in postmenopausal women significantly increases the risk of gallbladder diseases [118, 119]. Interestingly, this risk is lower with a transdermal therapy that with oral therapy [120].

In 2004 Sumi et al. [121] reported that Estrogen Receptor β (ERβ) expression was significantly reduced in the cancerous regions of gallbladder cancers and was completely lost at the invasive front. Loss of ERβ was associated with malignant properties of the primary tumor such as lymph node metastasis, advanced stage, lower differentiation of histological type, lymphatic invasion and a poor prognosis of the patients. ERβ is the nuclear receptor which has antiproliferative actions in many animal models including cancer cell lines [122, 123] and tumor xenographs [124, 125].

Very little is known about the molecular and genetic pathways of gallbladder cancer. Unlike in many other cancers, Ras and p53 genes do not appear to cooperate in gallbladder cancer [126], while the cyclin-dependent kinase 4 inhibitors p16Ink4/CDKN2, p16Ink4 and p15Ink4B are involved [127].

2 AIMS OF THE THESIS

The overall aim of this thesis is to determine the specific and distinct roles of LXRβ by studying the phenotype of LXRβ-/- mice in comparison with LXRα-/- and LXRα-/-β-/- mice.

2.1 PAPER I

As described previously [128, 129], by the age of 7 months, LXRβ-/- male mice are affected by a progressive death of big motor neurons in the latero-ventricular horn of the spinal cord.

β-sitosterol, a compound structurally similar to cholesterol, has been shown to increase the expression of LXR target genes [39]. β-sitosterol is known to be toxic to motor neurons and it is also thought to be one of the environmental factors that in concert with unknown genetic predispositions could lead to the ALS-PDC in Guam population [130].

Aim of this study was to investigate the possible toxicity of β-sitosterol in LXRβ-/- mice with particular attention to:

- Motor coordination;
- Intestinal expression of ABCG5, ABCG8 transporters;
- Histopathology of spinal cord and substantia nigra, two areas involved in ALS-PCD complex;
- Cholesterol levels in brain and serum.

2.2 PAPER II

As previously discussed, LXRβ-/- mice demonstrate a reduction in the size of perigonadal fat pad that is characterized by smaller adipocytes, compared to WT mice [63]. LXRβ-/- mice are thinner and resistant to weight gain when fed with a diet containing a high amount of fat. A similar phenotype has also been described in LXRα-/-β-/- mice but not in LXRα-/- mice indicating a specific role of LXRβ in controlling body weight [63, 131, 132].

Malabsorption syndrome is a clinical condition characterized by weight loss or growth failure in children that results from unsuccessful nutrient absorption from the diet. One of the main causes of malabsorption syndrome is a pancreatic exocrine insufficiency [109].

Aim of this study was to investigate the pathogenesis of the lean phenotype in LXRβ-/- mice with particular attention to the:

- Pancreatic exocrine function (assay of serum amylase, lipase, fecal protease);
- Histopathology of the pancreas;
- Transmission Electron Microscopy (TEM) of the pancreas.

2.3 PAPER III

According to the findings of Paper II (see Results section), the water channel Aquaporin-1 (AQP-1) appears to have a reduced expression (protein and mRNA) in the pancreas of LXRβ-/- mice.

The gallbladder, the storage organ for bile, is one of the most active water transporting organs of the digestive system. Two aquaporins have been identified in the cholangiocytes of this tissue: AQP-1 and AQP-8.

Aim of this study was to investigate whether in the gallbladder of LXRβ-/- mice water transport was affected with particular attention to:

- mRNA and protein expression of AQP-1 and AQP-8 in LXRβ-/-mice;
- mRNA and protein expression of AQP-1 and AQP-8 in WT mice treated with LXR agonist;
- histopathology of the gallbladders.

2.4 PAPER IV

According to the findings in Paper III (see Results section), the male LXRβ-/- mouse gallbladder does not show any macroscopic alterations. Gallbladder diseases are two to six times more

frequent in women that in man and the incidence is even higher in patients undergoing hormonal replacement therapy [118].

Aim of this paper was to study the phenotype of female LXRα-/-, LXRβ-/-, LXRα-/-β-/- gallbladders in particular with reference to:

- Histopathology;
- Proliferation/cell death markers;
- Morphology after ovariectomy.

3 NOTES ON METHODOLOGY

Material and methods used in this thesis are fully described in the specific section of each paper. They include:

- Knock-out animals;
- Immunohistochemistry;
- Western blotting;
- RT-PCR real time;
- Cholesterol assay in brain;
- Preabsorption of LXRβ anitibody;
- Treatment of mice with LXRs agonists;
- Evaluation of motor function with rotating rod test;
- Amylase and lipase assays;
- Total protease assays;
- Electron microscopy (TEM, iEM);
- ELISA

4 RESULTS

4.1 PAPER I

Evaluation of motor coordination, measured as retention time on a rota-rod, confirmed the motor disability of LXRβ-/- mice already evident from 5 months of age. Administration of β-sitosterol did not affect motor function in WT mice at any age but markedly worsened the disability of LXRβ-/- mice so that, at 16 months of age, they were almost paralyzed.

The histopathological study of the lateroventricular horn of the spinal cord (L1) showed a drastic reduction in the number of motor neurons in LXRβ-/- mice after treatment with β-sitosterol. In the few motor neurons left in LXRβ-/- mice, it was possible to localize cytoplasmatic inclusions positive for TDP-43 and ubiquitin, typical feature of ALS.

Analysis of the substantia nigra demonstrated that dopaminergic neurons of β-sitosterol-treated-LXRβ-/- mice were shrunken with reduced number of projections. Besides, the number of activated microglia was higher in the pars reticulata of LXRβ-/- mice treated with vehicle or β-sitosterol. These data demonstrated that when LXRβ signaling is abnormal, ingestion of β-sitosterol can damage both the spinal cord and the substantia nigra as is seen in ALS-PDC.

To evaluate if a decreased intestinal clearance of β-sitosterol could lead to an increase in its uptake, mRNA levels of ABCG5/G8 and serum levels of β-sitosterol were determined. Interestingly, these parameters were unaffected by either LXRβ deletion or β-sitosterol treatment, indicating a main role of LXRα in controlling these transporters.

Moreover, brain levels of cholesterol were decreased both in WT and in LXRβ-/- mice after β-sitosterol treatment suggesting a role for β-sitosterol in inducing cholesterol clearance from the brain, probably through LXRα activation.

4.2 PAPER II

Anthropometric measurements showed that both male and female LXRβ-/- mice had significantly lower body weights, weight/length ratios and perigonadal fat pad size than WT mice. Interestingly, the caloric intake monitored for 1 week was not different between the four genotypes in male mice, whereas a significant increase in the caloric intake was detected in LXRβ-/- female mice that despite the augmented caloric intake, did not gain weight.

Evaluation of pancreatic function demonstrated a reduced activity of α-amylase and lipase in serum of both LXRβ-/- male and female mice, compared to WT. Total protease in the gut, considered an indirect index of pancreatic secretion, was measured using Azo-casein as a substrate. A drastic reduction of proteolytic activity in the feces was evident in the transgenic animals of both sexes.

Histopathological analysis of LXRβ-/- pancreas showed a massive inflammatory infiltrate around large and medium-size pancreatic ducts that exhibited a high rate of cell death, without any compensatory proliferation, in the ductal epithelium. Electron microscopic studies of the transgenic pancreas revealed a dilatation of the ducts with dense intra-ductal material, including “scroll-like” structures commonly seen in cystic fibrosis.

With specific antibodies, we studied the expression of LXRβ and the water channel, aquaporin-1 in the ductal epithelium of the pancreas. In wild type mice, ductal epithelial cells expressed LXRβ in the nuclei and aquaporin-1 on the plasma membrane. Interestingly, aquaporin-1 was almost undetectable on the luminal surface of pancreatic duct epithelial cells and, in parallel, mRNA levels of aquaporin-1 were reduced in LXRβ-/- mice, compared to WT.

To test the possibility that AQP-1 could be a target gene of LXRβ, WT mice were treated with LXR-agonist (T0901317) for 7 days; after treatment, levels of AQP-1 mRNA were significantly higher than those in vehicle-treated mice indicating that AQP-1 expression is regulated by LXRβ.

4.3 PAPER III

Expression of AQP-1 and AQP-8 in the gallbladder was studied with RT-PCR and with immunohistochemistry. In LXRβ-/- gallbladders, mRNA levels of both AQP-1 and AQP-8 were significantly reduced compared to WT and their characteristic immunoreactivity on the plasma membrane (apical-baso-lateral for AQP-1 and apical for AQP-8) was not detectable as in WT mice. The pattern of expression of water channels in LXRα-/- gallbladders was similar to WT indicating a possible specific role of LXRβ in controlling AQP-1 and AQP-8. Moreover, treatment of WT mice with LXR agonist (T0901317) induced the gene expression of water channels as well as the cholesterol transporters ABCG5/G8, known target genes of LXR, in the gallbladder. Also the immunoreactivity of AQP-1 was much stronger in treated WT gallbladders. Administration of LXR agonist to LXRβ-/- mice did not lead to any increase in protein expression of AQP-1, detected and quantified by iEM.

Morphologically, gallbladders of LXRβ-/- and WT mice had similar volume and gross anatomy while WT-LXR-agonist treated mice showed a fasted reduced gallbladder volume with the presence of abundant cholesterol crystals in the bile.

At microscopic level, LXRβ-/- cholangiocytes showed a loss of cell polarity and at TEM level a conical shape with abundant electron-dense material together with osmiophilic lamellar bodies in dilated extracellular spaces.

4.4 PAPER IV

At the age of 11 months, LXRβ-/- female mice were affected by a severe gallbladder disease: a wide range of preneoplastic lesions like dysplasia, metaplasia, hyperplasia and adenomas were detectable. These lesions degenerated to cancer that was evident in 19 months old mice. Assessment of proliferation, performed with PCNA staining, showed an increased proliferation rate in LXRβ-/- mice starting from 4 months, when, interestingly, the gallbladder morphology was normal. A compensatory increased cell death rate, studied with TUNEL staining, was detectable at this age, but it decreased markedly when the preneoplastic lesions developed.

Surprisingly, the gallbladders of female and male LXRα-/- and LXRα-/-β-/- mice as well as male LXRβ-/- mice were unaffected.

Mechanistically, TGFβ signaling seems to be involved in the neoplastic gallbladder phenotype since increased expression of down-stream genes of TGFβ (pSMAD2-3), leading to loss of E-cadherin, was evident in LXRβ-/- female gallbladders.

The prevalence of gallbladder disease only in female mice together with the high incidence of the disease in women [115] motivated a study regarding a possible role of sexual hormones in the interplay between LXRβ and TGFβ. LXRβ-/- mice were ovariectomized at 3 months. Surprisingly, at 12 months of age, no morphological alterations were detectable in the gallbladders of ovariectomized LXRβ-/- mice and TGFβ signaling was reduced as in WT mice.

5 DISCUSSION

Results of **paper I** demonstrated that in LXRβ-/- mice, ingestion of β-sitosterol has marked neurodegenerative consequences both in the spinal cord and in the substantia nigra, resembling a phenotype similar to ALS-PDC.

Although LXRs are involved in overall cholesterol homeostasis, blood cholesterol levels were not affected either by LXRβ deletion or β-sitosterol treatment. However, LXRβ-/- mice were characterized by lipid inclusions in motor neurons of the spinal cord and high cholesterol levels in the brain. After β-sitosterol treatment, there was a decrease in brain cholesterol levels while in the spinal cord it was difficult to evaluate changes in lipid inclusions, because of low number of motor neurons left.

We interpret this data to mean that β-sitosterol, an activator of both LXRα and LXRβ, stimulates LXRα in LXRβ-/- mice promoting cholesterol excretion from the brain. Further evidence of an increase in cholesterol elimination from the brain is the high level of brain 24-hydroxycholesterol in β-sitosterol-treated LXRβ-/- mice.

Maintenance of appropriate cholesterol balance in the brain is crucial for many signal pathways like synaptic vesicle turnover, function of calcium channels, neurotransmitter release, signaling of GABA and glutamate. Our studies show that when cholesterol levels are affected in either direction, mice demonstrate a neurological phenotype (table 3).

	LXRβ-/- mice	**LXRβ-/- mice + β-sitosterol**
Blood	*no difference vs WT*	*no difference vs WT* *no differences vs vehicle*
Brain	*higher vs WT*	*lower vs WT (ns)* *lower vs vehicle*
Spinal cord (lipid inclusions)	*higher*	*only few motor neurons left*
Pnenotype	*ALS*	*ALS-PDC*

Table 3: *summary of the main cholesterol imbalances in LXRβ-/- male mice.*

Paper II demonstrates that pancreatic exocrine function is severely affected in LXRβ-/- mice, as shown by low levels of serum amylase and lipase, low levels of fecal total protease, massive infiltration of immune cells all around pancreatic ducts with increased cell death of the ductal epithelium and dense secretion obstructing the lumen of intralobular ducts. The cause of dense pancreatic secretions seems to be the reduced expression of AQP-1 on the luminal surface of pancreatic ductal epithelial cells. AQP-1 is a water channel protein with a key role in trans-cellular fluid transport. AQP-1-/- mice demonstrate mild growth retardation on standard diet [135] and, when fed with a high-fat diet, they are resistant to weight-gain, develop steatorrhea and have a decreased concentration of amylase and lipase in the pancreatic fluid [136]. It seems that defective secretion of water in the pancreatic ducts leads to a modification in the composition of pancreatic juice that damages the pancreatic epithelia and finally leads to exocrine insufficiency.

In the digestive system AQP-1 is expressed in endothelial cells of capillaries, small vessels and lymphatic capillaries of the small intestine [137], in cholangiocytes of liver, bile ducts [138] and gallbladder [139] and in the inter- and intralobular pancreatic ducts [140] where it seems to participate in bile and pancreatic juice formation.

In **paper III** the expression of AQPs is further investigated in the gallbladder, one of the most active water-transporting organs of the digestive system. LXRβ-/- cholangiocytes show a markedly reduced expression of AQP-1 and AQP-8 both at mRNA and protein levels while LXR-activation with synthetic ligand increases their expression in WT animals but not in LXRβ-/- mice. Morphologically, in male LXRβ-/- mice, the reduced AQP-1 expression in gallbladder cholangiocytes is associated with thinner gallbladder wall, loss of cell polarity and accumulation of osmiophilic lamellar bodies in the extracellular spaces.

The lack of increase in AQP-1 content in LXRβ-/- mice treated with LXR agonist, together with the normal profile of the same AQPs in LXRα-/- mice indicates that specifically

the β isoform of LXR may be the transcriptional controller of water channels in the gallbladder and pancreas.

In support of this notion is the fact that LXRβ-/- mice share important phenotypical characteristics with AQP-1-/- mice: resistance to gain weight [136, 141], a severe polyuria (Gabbi C. unpublished results) and alterations in the skin [142], testicles [143-145], lungs and salivary glands (Gabbi C. unpublished results) that could be explained at least in part by a defective water transport. Indeed, AQP-1, expressed in the kidney proximal tubule, descending limb of Henle, and in vasa recta, is a key player in water reabsorption from the urine explaining the severe polyuria and inability to concentrate urine in AQP-1-/- mice [146, 147] and in LXRβ-/- mice (Gabbi C, unpublished results).

In the CNS, strong expression of AQP-1 has been described on the luminal surface of the choroid plexus epithelium [148, 149], the main site of production of cerebrospinal fluid (CSF). This fluid not only provides physical support in the CNS but also facilitates transport of nutrients in the subarachnoid space surrounding the brain and the spinal cord [150]. In mice lacking AQP-1 there is a 25% reduction in CSF production, compared to WT [151]. We may speculate that there could be a reduction in AQP-1 expression in the choroid plexus of LXRβ-/- mice. Such a reduction would lead to electrolyte imbalances and nutrient deficiencies and could be the one of the causes of the pathogenesis of neurodegenerative diseases in LXRβ-/- mice.

Female LXRβ-/- gallbladders, studied in **paper IV**, are characterized by increased cell proliferation at the age of 4 months, by the presence of numerous pre-neoplastic lesions (adenomas, dysplasia, metaplasia) at the age of 11 months, degenerating to carcinomas at 19 months.

Carcinogenesis of the gallbladder, estimated to occur within a time frame of 15 years in humans [152], is a long process in which a concert of numerous “hits” participates in the neoplastic transformation of the epithelium [115]. In LXRβ-/- female mice several hitting factors have been identified. First seems to be an increased inflammatory reaction, evident

histologically from the constant background of cholecystitis in the preneoplastic lesions of LXRβ-/- gallbladders. LXRβ has been described to have a potent anti-inflammatory activity [55] by transrepressing the NF-*k*B signal, therefore, its absence induces a cascade of NF-*k*B mediated events that may trigger inflammation and drive it into cancer [153, 154]. Interestingly, in humans, infection with numerous microbial agents (*S.typhi, H. bilis, H. hepaticus, E.coli*) has been described in association with gallbladder cancer [155]. Besides, LXRα-/-β-/- mice have been shown to be more susceptible to infections [156]. Speculatively, bacteria may not only induce inflammation directly but also, by activating TLR3/4 [70], be responsible for an inhibition of LXR activity and therefore reinforcing an inflammatory reaction.

Another factor in gallbladder carcinogenesis in LXRβ-/- mice is a complex interplay between TGFβ and estrogens. Downstream genes of TGFβ appear to be upregulated in LXRβ-/- female transformed gallbladders and, surprisingly, ovariectomy prevents the development of pre-neoplastic lesions and reduces TGFβ signaling. Despite strong epidemiological data in humans (high gallbladder cancer incidence in females and a positive association with HRT) [115] indicating a crucial pathogenetic role for estrogens, as well as a correlation of TGFβ polymorphism with gallbladder cancer [157], many aspects of this interplay remain unclear. To be considered also that although in LXRβ-/- female mice, no differences in the expression of ERα and ERβ proteins have been detected compared to WT, an imbalance in their activity, influencing LXRβ/TGFβ interplay may not be excluded.

Moreover, a direct action of LXRβ on cell cycle control, as shown in several cell culture systems [158] has to be considered in the transformation of LXRβ-/- gallbladder epithelium.

The contribution of AQP-1 in carcinogenesis should also be considered. Indeed AQPs, are not only mediators of water transport but they are also involved in cell adhesion and migration [159] with an emerging role in tumorigenesis and metastasis formation [160, 161]. AQP-1 expression is affected in numerous human cancers; in particular in cholangiocarcinoma,

AQP-1 appears to be downregulated and its low expression correlates with poor prognosis, higher tumor size and lymph node metastases [162].

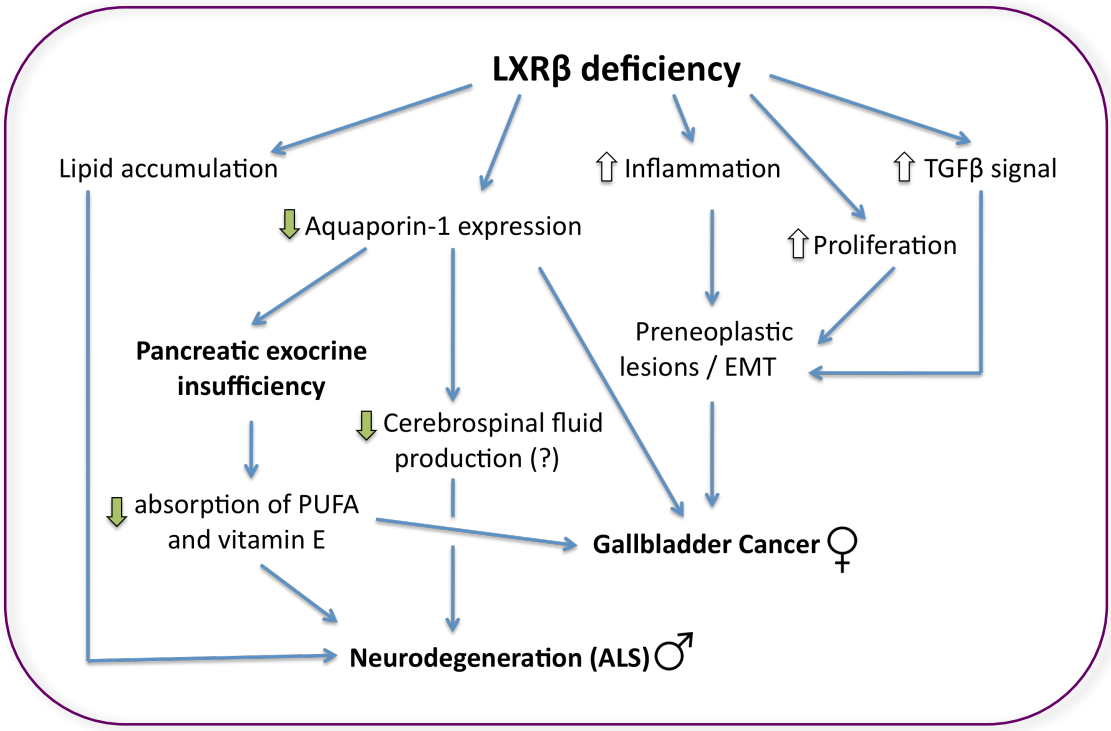

Figure 3. *Hypothesis for the cascade of events following LXRβ deficiency. Knocking-out LXRβ in mice leads to (i) cholesterol accumulation in the big motor neuron of the spinal cord that contributes to neurodegeneration in male mice; (ii) reduced aquaporin-1 expression that is responsible for a pancreatic exocrine insufficiency, malignant transformation of gallbladder cholangiocytes, reduced CSF production; (iii) increased (iv) inflammation, (v) proliferation and (vi) TGFβ signalling that represent multiple "hits" in the carcinogenesis of the female gallbladders.*

Several studies have examined the roles of nutrients as environmental factors that, in concert with genetic predisposition, could contribute to the pathogenesis of ALS and gallbladder cancer. Interestingly, a premorbid daily intake of n-3 polyunsatured fatty acids (PUFA) and vitamin E has been shown to be significantly lower in patients with ALS [163, 164] and Parkinson´s disease [165]. In addition, low levels of vitamin E and other antioxidative vitamins have been detected in patients affected by gallbladder cancer [166, 167]. Indeed n-3 PUFA, acting as substrate in the synthesis of prostaglandins with anti-inflammatory effects has anti-inflammatory [168], antineoplastic [169] and neuroprotective actions [170]. Vitamin E is an antioxidant agent that prevents lipid peroxidation and acts as a neuroprotective factor both in humans [171] and in animal models of ALS [172].

During pancreatic exocrine insufficiency, the lack of pancreatic lipolytic enzymes in the intestinal lumen affects the absorption of lipids, in particular triglycerides, from the diet leading to a reduced uptake of PUFA and vitamins (as vitamin E) that require lipid micells to be absorbed. Interestingly, although no primary pancreatic involvement in ALS has been described, it has been shown that patients affected by ALS, have a reduced exocrine pancreatic function in particular after secretion stimulation [173].

We speculate (Figure 3) that in LXRβ-/- male mice, the pancreatic exocrine insufficiency, which appears at an early age, could be responsible of a lack in n-3 PUFA and vitamin E. These deficiencies could lead to vulnerability to oxidative stress and inflammation and in turn contribute to the pathogenesis of ALS in males and gallbladder cancer in female LXRβ-/- mice.

6 CONCLUSIONS AND PERSPECTIVES

The articles in this thesis open up completely new perspectives in the specific pathophysiological activity of the oxysterol receptor LXRβ in controlling not only cholesterol homeostasis in central nervous system but also water channels in pancreas and gallbladder and carcinogenesis in female gallbladders.

The observation that LXRβ is essential in maintaining the physiological response to β-sitosterol administration, suggests that LXRβ dysfunction could be a genetic predisposition that, in the Guam population, in concert with environmental factors like phytosterols, participates in the pathogenesis of ALS-PDC.

The specific transcriptional control of water channels by LXRβ in pancreatic ductal epithelial cells and gallbladder cholangiocytes, leads to a new perspective on LXRβ function in diseases associated with a dysregulation of the gastrointestinal fluid balance such as in pancreatic insufficiency or cystic fibrosis.

More studies are required to investigate the mechanism of transcriptional control of LXRβ over AQPs focusing in particular on the identification of possible LXR-binding sites on AQP genes. A crucial factor that remains to be investigated is the role of sexual hormones in influencing LXRβ activity, given that only LXRβ-/- male mice are affected by ALS and only LXRβ-/- female mice present the gallbladder carcinogenesis that is prevented by ovariectomy.

Further studies are planned in order to identify the role of LXRβ in human diseases in particular in ALS, chronic pancreatitis and gallbladder cancer.

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