LXRα in the ageing process

Control of hepatic glucose and lipid metabolism

Christian Bindesbøll

Master Thesis
Department of Nutrition Research
Institute of Basic Medical Sciences

UNIVERSITY OF OSLO
March 2008
# Contents

CONTENTS ........................................................................................................................................3

ACKNOWLEDGMENTS ................................................................................................................ ....5

ABBREVIATIONS ..........................................................................................................................7

SUMMARY .......................................................................................................................................10

1. INTRODUCTION ................................................................................................................... .12
   1.1 AGEING AND DISEASE: GLUCOSE AND LIPID METABOLISM .....................................................12
       1.1.1 Metabolism ..................................................................................................................13
       1.1.2 Insulin, glucose and lipid metabolism ........................................................................13
   1.2 NUCLEAR RECEPTORS ............................................................................................................15
       1.2.1 Liver X Receptors ........................................................................................................22
   1.3 THE LIVER ..............................................................................................................................27

2. OBJECTIVES ..........................................................................................................................31

3. MATERIALS ............................................................................................................................33

4. METHODS ...............................................................................................................................35
   4.1 ANIMAL EXPERIMENTS ...........................................................................................................35
   4.2 RNA ANALYSIS ......................................................................................................................36
       4.2.1 DEPC-water ................................................................................................................37
       4.2.2 Isolation of total RNA from liver tissue ......................................................................37
       4.2.3 cDNA synthesis ...........................................................................................................39
   4.3 ESSENTIAL RNA - DNA TECHNIQUES ....................................................................................40
       4.3.1 Real time polymerase chain reaction .........................................................................40
   4.4 SERUM ANALYSIS ................................................................................................................46
4.4.1 Glucose Quantification .......................................................... 46
4.4.2 Insulin Quantification ........................................................... 47
4.4.3 Lipid Quantification ............................................................. 48
4.5 Statistics .................................................................................. 48

5. RESULTS .................................................................................... 50
5.1 Animal characteristics in female and male WT and LXRA⁻/⁻ mice, aged 1-9 months 50
5.2 Hepatic expression of selected genes in ageing mice (1-9 months) .................. 52
5.2.1 Genes involved in hepatic glucose metabolism .................................... 53
5.2.2 Genes involved in hepatic cholesterol metabolism ............................... 57
5.2.3 Genes involved in hepatic lipid metabolism .......................................... 59
5.2.4 Other metabolic genes ..................................................................... 62
5.2.5 Sex differences ............................................................................. 64

6. DISCUSSION .............................................................................. 66
6.1 Use of animals as a model system .................................................. 66
6.2 Methodology ........................................................................... 67
6.3 The role of LXR in ageing .......................................................... 69

7. CONCLUSION ............................................................................ 81
7.1 Future perspectives ................................................................... 82

8. REFERENCE LIST ..................................................................... 85
Acknowledgments

This work was conducted at the Department of Nutrition, University of Oslo, in the laboratory of Professor Hilde Irene Nebb.

I would like to express my gratitude to my supervisor Hilde Irene Nebb for welcoming me in your group, for introducing me to nutrigenomics, for your enthusiasm and support through hard times. Special thanks to my co-supervisor Sverre Holm for your detailed knowledge, enthusiasm and inspiration. I am greatly thankful for your support, educational discussions and challenges you have provided me with. Thanks to Borghild Arntsen, Yan Qin, Maria Nygård and all other members of Nebb’s research group for your helpful advices and fruitful discussions.

I would also like to give sincere thanks to Ferdinand Diener for encouragement and support, my friends outside the world of nutrition and my fellow students; Hanna Ræder, Ole Berg, Anne Høidalen and Cathrine Strandskogen for being good company.

Finally, I would like to thank my family for always believing in me.

Oslo, March 2008

Christian Bindesbøll
Abbreviations

11β-HSD-1  11β-Hydroxysteroid Dehydrogenase Type 1
36B4     Acidic Ribosomal Phosphoprotein PO
ABC      ATP-Binding Cassette
ABCG1    ATP-Binding Cassette Transporter G-1
ABCG8    ATP-Binding Cassette Transporter G-8
ACC      Acetyl CoA Carboxylase
apoE     Apolipoprotein E
ATP      Adenosine Triphosphate
BMAL     Brain and Muscle Aryl Hydrocarbon ARNT-like Protein
CETP     Cholesterol Ester Transfer Protein
ChREBP   Carbohydrate Response Element-binding Protein
CoA      Coenzyme A
CVD      Cardiovascular Disease(s)
CYP7A1    Cholesterol 7 α-hydroxylase
DBD      DNA Binding Domain
DEPC     Diethyl-Pyro-Carbonate
DNA      Deoxyribonucleic Acid
DPE      Downstream Promoter Element
DR       Direct Repeat
ELOVL    Fatty Acid Elongase
ER       Estrogen Receptor
FA       Fatty Acid
FAS      Fatty Acid Synthase
FBP-1    Fructose-1,6-bisphosphatase 1
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GCK</td>
<td>Glucokinase</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose Transporter</td>
</tr>
<tr>
<td>GTFs</td>
<td>General Transcription Factors</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone Acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>HMGCS1</td>
<td>3-Hydroxy-3-methylglutaryl Coenzyme A Synthase</td>
</tr>
<tr>
<td>HNF</td>
<td>Hepatocyte Nuclear Factor</td>
</tr>
<tr>
<td>INK-4</td>
<td>Inhibitor of Cyclin-Dependent Kinase 4</td>
</tr>
<tr>
<td>IR</td>
<td>Inverted repeat</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand Binding Domain</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein Lipase</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X Receptor</td>
</tr>
<tr>
<td>LXRα⁻/⁻</td>
<td>LXRα Knockout</td>
</tr>
<tr>
<td>LXRα⁻/⁻β⁻/⁻</td>
<td>LXRαβ double-knockout</td>
</tr>
<tr>
<td>LXRE</td>
<td>LXR Responsive Element</td>
</tr>
<tr>
<td>Min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated Fatty Acid</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non Esterified Fatty Acid</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate Dehydrogenase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate Carboxykinase</td>
</tr>
<tr>
<td>PLTP</td>
<td>Phospholipid Transfer Protein</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator Activated Receptor</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnancy X Receptor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RE</td>
<td>Response Element</td>
</tr>
<tr>
<td>RIP140</td>
<td>Receptor-interacting Protein 140</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotation per minute</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoic X Receptor</td>
</tr>
<tr>
<td>SCD-1</td>
<td>Stearoyl-CoA Desaturase 1</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic Nuclei</td>
</tr>
<tr>
<td>Sec</td>
<td>Second(s)</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol Regulatory Element Binding Protein</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA Box Binding Protein</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription Factor</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription Start Site</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
</tr>
<tr>
<td>WAT</td>
<td>White Adipose Tissue</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ZT3</td>
<td>Zeitgeber Time 3</td>
</tr>
</tbody>
</table>
Summary

Ageing is a complex physiological process shaped by nutrition, metabolism and hormones, with insulin, responsible for glucose absorption from the blood, being an important pro-ageing factor. The metabolic decline observed in ageing is characterized by changes in insulin action and body fat distribution, predisposing to diseases (atherosclerosis, coronary vascular disease [CVD], hyperlipidemia, obesity, insulin resistance and type 2 diabetes mellitus [T2DM]).

Liver X receptors (LXRs) are nuclear receptors (NRs) that are important in regulating cholesterol, lipid and glucose metabolism. Despite the wealth of data supporting the role of LXRs in energy metabolism, the information is scarce regarding how they integrate multiple inputs in whole organisms across life span.

The present study was conducted both to investigate how LXRα affects the regulation of energy metabolism in a physiological setting and to study sexual dimorphism in ageing mice. As such, wild type (WT) and LXRα−/− C57BL/6 mice, both females and males, were included. They had free access to food and water throughout the experiment and were sacrificed at either 1, 3, 6 or 9 months of age. The relative mRNA expression levels of hepatic genes were revealed using qRT-PCR, and several components in serum were measured. Our data demonstrate that, in the presence or absence of LXRα, several hepatic genes are regulated in an age dependent and/or sex dependent fashion. From the results of this thesis, we suggest that several hepatic LXRα target genes are regulated during the ageing process, such as the de novo lipogenic genes (fatty acid synthase [FAS] and stearoyl-CoA dehydrogenase-1 [SCD-1]). Their mRNA expression levels at 1 month are dependent upon LXRα. A gradual increase in the expression of these genes, occur in LXRα−/− animals aged 1-9 months, indicating that other mechanisms/proteins come into play to compensate for the loss of LXRα. Furthermore, this is also the first report assessing the link between LXRα and circadian gene regulation. Our data show that LXRα is important in regulating the basal mRNA expression of the circadian gene, BMAL1, in 1 month old animals.
It is therefore tempting to speculate that LXR\(\alpha\) is involved in circadian signaling pathways. In addition, this study also reveals many differences between female and male mice, both in plasma glucose concentration, serum levels (\textit{i.e.} insulin, triacylglycerol (TAG), cholesterol) and relative mRNA levels (\textit{i.e.} ATP-binding cassette [ABC] cholesterol transporters ABCG5/G8, cholesterol 7 \(\alpha\)-hydroxylase [CYP7A1], Fructose-1,6-bisphosphatase 1 [FBP-1], glucose transporter [GLUT] 2).

In order to further evaluate the potential role of LXR\(\alpha\) in the ageing process through glucose and lipid metabolic control, the crosstalk with other tissues/organs, NRs, hormones and cofactors must be addressed, as no single pathway is believed to function in an isolated manner. Furthermore, it remains to be elucidated if similar differences are present in humans.
1. Introduction

1.1 Ageing and disease: Glucose and lipid metabolism

Although the term “ageing” is generally understood in broad terms, the ageing process is extremely complex and multifaceted. Ageing include many processes, interactive and interdependent, that determine lifespan and healthspan. Lifestyle and other environmental factors can profoundly influence the ageing process and its impact on the quality of life. The complexity of molecular and physiological changes in the ageing process is not fully elucidated. As such, no single definition of ageing is universally accepted. Nevertheless, from a biological perspective, ageing can defined as the progressive loss of physiological functions that increase the probability of death (1). Other than heredity, ageing is shaped by nutrition, metabolism and hormones with insulin, the hormone responsible of glucose absorption from the blood, as an important pro-ageing factor (2;3). Several changes normally occur with increased life span, including a progressive decrease in most, if not all physiological functions, atrophy of most organs, an increased vulnerability to infections, trauma and various immune abnormalities (i.e. autoimmune disorders, lymphoproliferative disorders, amyloidosis) and an increased susceptibility to malignancy (2). The loss of physiological function occurs both within individual cells and within the organism as a whole. In addition, the ageing process is characterized by a metabolic decline leading to altered distribution of body fat, changes in glucose and lipid metabolism and changes in insulin signaling (4-6). Also, insulin sensitivity is normally decreased during ageing (7-12), and insulin resistance is an important risk factor (13;14) for a variety of illnesses that affect morbidity and mortality among the elderly (15;16). However, not all studies show a positive correlation between insulin resistance and ageing (17;18), and some studies show that insulin sensitivity is unchanged in healthy subjects with increased age (19;20). Together, the abovementioned metabolic factors affect life span both in invertebrates and in mammals. It is known that dysregulated
glucose and lipid metabolism increase the risk for several age-related diseases (i.e. insulin resistance and T2DM (21), atherosclerosis, hyperlipidemia and obesity, all of which can precipitate to cardiovascular (22) and neuropsychiatric diseases (23)). NRs function throughout development and ageing as molecular integrators of physiological regulations, such as growth, reproduction and metabolism. Multiple NRs, including the LXRs, are involved in the control of glucose and lipid metabolism (24;25). This makes them potential targets for treatment and prevention of age-associated diseases. Despite the critical role of LXRs in glucose and lipid metabolism, the current knowledge regarding how they integrate multiple inputs in whole organisms across life span is fragmental.

1.1.1 Metabolism

Metabolism is the sum of all physical and chemical processes by which components of living organisms are produced and maintained (anabolism), and also the transformation by which energy is made available for the uses of an organism (catabolism) (26). The energy in nutrients can be converted into energy in the organism and used for activity, growth, development and reproduction. Metabolic pathways involved in processing carbohydrates, lipids and amino acids are quantitatively the most important in energy metabolism. Each pathway is composed of multienzyme sequences, and each enzyme may exhibit important catalytic or regulatory features. In living organisms and cells these metabolic pathways intersect, forming an integrated and purposeful network of chemical reactions. The complex control of each pathway by hormones, transcription factors (TFs) and other components is not yet fully understood.

1.1.2 Insulin, glucose and lipid metabolism

During evolution mammals have evolved to maintain energy balance under various nutritional conditions. However, this ability is ill-suited to the lifestyles of modern society, as evidenced by the fact that obesity has reached epidemic proportions in
many industrialized countries. The essence in energy homeostasis is the ability to sense the amounts of circulating nutrients and maintain tightly controlled nutrient levels by adjusting metabolic pathways. Many hormones and nutrients impinge on metabolic networks leading to homeostasis. Glucose and lipid metabolism are intimately linked through crosstalk with insulin as one of the most important hormonal factors (27). Insulin is a peptide hormone produced and secreted from pancreatic β-cells in response elevated glucose concentrations in plasma. Increased secretion of insulin occurs after consumption of glucose or a carbohydrate rich meal, leading to elevated plasma levels within minutes (28). Although glucose is the main stimulus to insulin secretion, amino acids and ketone bodies are also stimulators, albeit to differing extents. The main role of insulin is to keep glucose levels within a narrow range regardless of fluctuations in dietary glucose and lipid ingestion (29). Insulin has its most prominent effects in liver, muscle and adipose tissue (Figure 1.1). Glucose derived from diet or endogenous sources stimulates insulin secretion, which in turn, promotes glucose uptake by skeletal muscle and adipose tissue, inhibits fat lipolysis and opposes hepatic glycogenolysis and gluconeogenesis. Being one of the most important anabolic hormones, insulin promotes storage of carbohydrates, lipids and proteins, while inhibiting their degradation and release into the blood.

Diet and endogenous synthetic pathways provide the body with its requirement for lipids. The synthesis of fatty acids (FAs) and cholesterol can occur in any cell, and increases the multiple lipid compounds. This is especially important in liver and adipose tissue; organs specialized in lipid transport and storage. FA synthesis increases in response to elevated levels of plasma glucose, as seen in obesity and T2DM (30). Free FAs liberated from adipose tissue contribute to insulin resistance in the liver and skeletal muscle (27). The identification of key regulators in controlling expression of genes involved in glucose and lipid metabolism have revolutionized the understanding of the intimate link between these metabolic pathways. It is evident from numerous studies that the balance of levels of receptors, cofactors and ligand availability is fundamental in controlling energy homeostasis, as discussed later.
Figure 1.1. Tissue-tissue crosstalk between glucose and lipid metabolism. Glucose stimulates insulin secretion. Fat derived signals, including adiponectin, resistin and TNF-α, modulate insulin sensitivity and FA metabolism in muscle and liver. See text for more details. Adapted from (27).

1.2 Nuclear Receptors

In eukaryotes, transcription is performed by RNA polymerase- I, II or III (31). They exhibit similar structure and function, but transcribe different types of genes. RNA polymerase I and III transcribe genes encoding transfer RNA, ribosomal RNA and various small RNAs, whereas RNA polymerase II is responsible for transcription encoding proteins, the messenger RNAs (mRNAs). Most eukaryotic genes have coding sequences (exons) interrupted by noncoding sequences (introns). TFs regulate gene expression by binding to sequence specific sites (transcriptional regulatory domains) in the promoter of their target genes (32). These elements are often referred to as response elements (RE), cis elements or enhancer elements (Figure 1.2).
**Figure 1.2. A model of transcriptional control regulating gene expression.** A complex arrangement of multiple clustered enhancer modules interspersed with silencer and insulator elements which can be located 10-50 kb either upstream or downstream of a composite core promoter containing TATA box (TATA), Initiator sequences (INR) and downstream promoter elements (DPE). Adapted from (32).

The expression of genes may depend on RE upstream or downstream the transcription start site (TSS), but they are usually located immediately 5’ of the TSS. The complex regulation of genes involves several different sets of REs.

Different combinations of TFs expressed in various cell types ensure a strict control of gene transcription, ultimately leading to satisfy the needs of each cell (31).

NRs are primarily understood as intracellular TFs that directly regulate gene expression, generally in response to lipophilic molecules (33). They are part of a NR superfamily, describing an evolutionary conserved group of TFs, all of which share common structural features (24), despite variation in ligand sensitivity (Fig 1.3.A) (34).
Figure 1.3. The nuclear receptor superfamily. (A) Schematic structure of NRs. (B) Classification of NRs according to physiological properties and source and type of ligand. Adapted from (34).

Even before the first genes encoding NRs were cloned, it was known that they are modular proteins with three major domains (Figure 1.3.A) (35). The N-terminal domain contains a ligand-independent transcription function (AF-1). This transactivation domain is recognized by cofactors and/or other transcription factors. In the core, the highly conserved DNA binding domain (DBD) is located. It contains two zinc finger motifs responsible for targeting the receptor to its specific DNA responsive element (RE), located in the promoter of target genes (33;36). A large ligand binding domain (LBD), is located in the C-terminal half of the receptor. This domain directs specificity to a biological response by recognizing and binding specific hormonal and nonhormonal ligands, dimerisation, interaction with heat shock proteins, nuclear localization and transactivation. The LBDs of all NRs share a common three-dimensional structure (37). A variable length hinge region (D domain)
between the DBD and LBD permits a flexible three-dimensional structure, allowing simultaneous receptor dimerisation and DNA binding. Two nuclear localization signal sequences are found in the hinge and C-terminal region. Some NRs have an additional domain at the C-terminal (F-region). However, the function of this domain is not fully understood. When a ligand is associated to its receptor, the conformation is changed. This facilitates dissociation of corepressors, recruitment of coactivators and binding of the complex to the DNA in the promoter region of specific target genes (34). In the absence of a ligand, the LBD of many, but not all NRs (i.e. not steroid receptors) is bound to a set of transcriptional corepressors, which recruit transcriptional complexes that contain specific histone deacetylases (HDACs) (38). These deacetylases create a condensed chromatin structure over the gene promoter, leading to gene repression (Figure 1.4).
Figure 1.4. Nuclear receptors in action. After diffusion through the cell membrane, the ligand can bind to its cognate receptor where it can interact with kinases directly and thereby exert “non-genomic effects” (a). The ratio between cytoplasmatic and nuclear location can vary between different nuclear receptors and is influenced by the nature of the ligand. Ligand binding modulates the interaction of the receptor with numerical factors. In the absence of ligand, several NRs are believed to be bound to regulatory regions of the target genes as a corepressor or histone deacetylase (HDAC) complex (b). Histone deacetylation is responsible for the chromatin condensation that accounts for the gene-silencing effect of apo receptors. Ligand binding releases the HDAC complex (c) and leads to the recruitment of histone acyltransferases (HAT) and chromatin remodelling (CRM) complexes (d). The temporal order and requirement of these complexes can occur in a receptor-, target-gene- and cell-specific manner. In the final step (e), the polymerase II holoenzyme, which comprises the pol II enzyme, TAF (TATA binding protein-associated factor) and mediator complexes, is recruited and increase the frequency of transcription initiation. Adapted from (38).
There are NRs in all cells of our body. To this date, the humane genome is reported to contain 48 members of the NR superfamily (39). Depending on their physiological function and type of ligand, NRs can be subdivided into endocrine receptors, adopted orphan receptors and orphan receptors (Fig 1.3.B). The classic endocrine receptors mediate actions of steroid hormones, thyroid hormones and the fat soluble vitamins A and D (40). They bind ligands with high affinity. This subgroup is divided in two, the steroid nuclear hormone receptors (Fig 1.3.B; blue box) and receptors for thyroid hormone (TR), retinoic acid (RAR), vitamin D (VDR) and ecdysone (EcR) (Fig 1.3.B; purple box). They bind to the DNA as dimers; steroid receptors as homodimers, the rest heterodimerise with RXR. The steroid receptors include receptors for estrogen (ER), progesterone (PR), androgen (AR), glucocorticoid (GR) and mineralcorticoid (MR). They bind ligands synthesized from endogenous endocrine sources, to control reproduction, carbohydrate metabolism and electrolyte homeostasis.

The orphan NRs (Fig 1.3.B; black box) have no identified ligands. It is not known whether all orphan receptors have the ability to bind natural or synthetic ligands or whether they are true orphan receptors that do not contain a ligand-binding pocket and might be regulated by alternative mechanisms. Orphan receptors become adopted when they are shown to bind a physiological ligand.

Adopted orphan NRs (Fig 1.3.B; red box) heterodimerise with RXR. They bind dietary lipids with low affinity and act as lipid sensors in the body by maintaining lipid homeostasis through transcriptional control of genes involved in lipid metabolism, storage, transport and elimination. NRs compromised in this group are receptors for FAs (PPARs), oxysterols (LXR), bile acids (FXR) and xenobiotics (Pregnane X Receptor/ Steroid Xenobiotic Receptor [PXR/SXR] and Constitutive Androstane Receptor [CAR]). It should be emphasized that the response of a given NR to a particular ligand in a specific tissue is dictated by a number of other proteins interacting with this NR following ligand-induced allostERIC changes that generate, expose or remove interaction surfaces. The proteins that interact with the specific NR
may range from other NRs and transactivators to transcriptional cofactors. A number of transcriptional coactivators play important roles in the integration of metabolic processes, including peroxisomal proliferator coactivator (PGC)-1α, PGC-1β, steroid receptor coactivator 1 and transcriptional intermediary factor 2 (41;42). In addition, corepressors can regulate networks of metabolic genes. For example, receptor-interacting protein 140 (RIP140) promotes lipid storage in adipose tissue by inhibiting the expression of genes involved in mitochondrial biogenesis, oxidative phosphorylation, and FA oxidation (43-45). RIP140 interacts with several NRs, including peroxisome proliferator activated receptors (PPARs), estrogen-receptor related receptors (ERRs) and LXR that regulate metabolic pathways (46;47). RIP140 is important in regulating the function of LXR in vivo in two distinct ways; the induction of lipogenesis and the repression of gluconeogenic genes (48).

Most NRs bind to their specific RE as homo- or heterodimers with other members of the nuclear receptor family. The RE exists as half-sites (six nucleotide sequence) separated by variable length of nucleotides between direct or inverted half-site repeats (Fig 1.5). For instance, the thyroid hormone receptors (T3Rs) preferentially bind to two AGGTCA half sites orientated as direct repeats with a four-base spacer (DR4); retinoic acid receptors (RARs) bind to the same AGGTCA half sites, but orientated as a DR5; estrogen receptors bind to AGGTCA half sites orientated as an inverted repeat with a three-base spacer (INV3); and androgen receptors (ARs) recognize an INV3 orientation containing AGAACA half sites (33).
Figure 1.5. **Nuclear receptor binding to DNA.** A nuclear receptor dimer bound to the RE upstream of a target gene. Each receptor is represented as two domains: DNA binding and hormone binding. The RE shown here is composed of two AGGTCA half sites in a direct repeat separated by a spacer; different half-site sequences, and orientations select for binding of different nuclear receptors. Adapted from (33).

## 1.2.1 Liver X Receptors

Cholesterol and sterol homeostasis is an important regulatory pathway closely controlled by NR function. Pathologic levels of cholesterol in the blood are detrimental as they may lead to excessive accumulation in vessel walls, a condition which might precipitate to atherosclerosis and CVD. Liver X Receptors (LXRs) are major cholesterol sensors in the body and play an essential role in regulating cholesterol and FA homeostasis (49-51). They are crucial in regulating genes in different steps involved in reverse cholesterol transport (52;53), the process of removing cholesterol from the vessel wall and transporting it to the liver, and genes involved in bile acid biosynthesis and excretion (54;55).

LXRα (NR1H3 (Nuclear Receptor Nomenclature Committee, 1999) also described as RLD-1) was first isolated from rat liver and later human liver, hence the name Liver X Receptor (56;57). Later LXRβ (NR1H2, also described as OR-1/UR/NER/RIP15) was identified (58-61). Both isoforms are highly related and share 78% amino acid sequence in both the DBD and LBD. LXRα is predominantly expressed in
macrophages and in metabolic tissues, such as the liver, skeletal muscle, adipose tissue, small intestine and kidney, but a lower expression level is also seen in the spleen, adrenal and pituitary glands (56;57;62). LXRβ is ubiquitous (58-61). The LXR/RXR heterodimer binds to a DR4 (AGGTCA nnnn AGGTCA) LXR response element (LXRE) in the promoter of target genes (57;61). The LXRs are activated by endogenous levels of oxysterols, oxidised cholesterol derivatives, such as 22(R)-hydroxycholesterol, 27-hydroxycholesterol and 24(S),25-epoxycholesterol (51;63;64). Desmosterol, the direct precursor of cholesterol in the biosynthesis, has also been shown to activate LXR in vitro (65) and in vivo (66). Recently, it has been suggested that physiological concentrations of glucose expected in the liver bind and stimulate LXR with efficacy similar to that of oxysterols (67), suggesting that glucose is an endogenous LXR ligand. However, further evidence is needed to validate glucose’s effects on LXR, leaving glucose as an uncertain ligand to this date. Acetyl-Podocarpic Dimer (APD) (68), T0901317 (49) and GW3965 (69) have all been described as synthetic compounds with ability to activate LXRs. The two latter do not share the same ligand specificity for LXRs. It has been shown that T0901317 also functions as a high-affinity ligand for PXR. Induced expression of PXR target genes, such as the scavenger receptor CD36, is also seen upon T0901317 stimulation, a property not shared by the more specific LXR ligand GW3965 (70). Recently, the synthetic ligand, 15-ketosterol, has also shown to bind LXRs with efficacy similar to that of oxysterols (71). Furthermore, polyunsaturated FAs (PUFAs) are found to be competitive inhibitors of LXR ligands (i.e. oxysterols), antagonizing LXR activity by inhibiting LXR/RXR binding to the LXRE (72;73), while FAs are positive regulators of LXRα gene expression in cultured hepatocytes (74). LXR/RXR is a so-called permissive heterodimer, in that it can be activated by ligands for either LXR or RXR. A synergistic activation is obtained if ligands for both NRs are present.

LXRs regulate key aspects of cholesterol, FA and carbohydrate metabolism (30;75-77). The generation of mice devoid of LXRα (LXRα−/−) has revealed key roles of LXRs in lipid homeostasis. LXRs regulate expression of multiple genes involved in efflux, transport and excretion of cholesterol and the FA synthesis pathway, as well
as lipoprotein metabolism (78-80). The first gene described as a direct LXRα target, CYP7A1, is regulated in mice, but not in humans (81). CYP7A1 is the rate limiting enzyme responsible for conversion of cholesterol to bile acids in liver. In the liver, LXR is also involved in transcriptional control of ABCG5 and ABCG8 (82;83), ABC transporters implicated in the biliary cholesterol excretion. Induction of intestinal ABCA1, ABCG5/G8 expression upon LXR activation is thought to limit the efficiency of cholesterol absorption and hence to accelerate fecal cholesterol disposal (53). LXRα−/− mice exhibit a vast hepatic cholesterol accumulation due to impaired cholesterol and bile acid metabolism, ultimately causing defective liver function. Excess extra-hepatic cholesterol must be transported to the liver and excreted as cholesterol or bile acids into the bile, and ultimately into the gut. Furthermore, macrophages play an essential role in this reverse cholesterol transport as excess cholesterol may convert these cells into foam cells and thereby promote atherosclerosis. In macrophages, the ABC transporters, ABCA1/G1, are regulated by LXRs (81;84). These transporters are involved in transport of cholesterol and phospholipids from cells to extracellular cholesterol acceptors, notably the lipid-poor apolipoproteins apoA1 and apoE. Reverse cholesterol transport is correlated with levels of the lipoprotein HDL. ApoE is a component of HDL that is regulated by LXR in macrophages and adipocytes, further underlying the role of LXR in cholesterol metabolism (78;79). Taken together, these and other studies provide compelling evidence for LXR as an important cholesterol sensor in the body (63;80;81;85) and that it might enhance reverse cholesterol transport in a tissue-specific manner. Furthermore, cholesterol ester transfer protein (CETP) (86) and phospholipid transfer protein (PLTP) (87) compromise lipid transfer proteins regulated by LXRs. CETP transports cholesteryl esters from HDL to the apolipoprotein B-100-containing lipoproteins very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and low density lipoprotein (LDL) (88). This leads to cholesteryl ester clearance when IDL and LDL particles are taken up by the liver. PLTP, in turn, is involved in the generation of efficient acceptors of cellular cholesterol (preβ-HDL) by transferring excess lipoprotein surface phospholipids (surface remnants) to the lipid-poor apoA-1. The remnants are generated when
lipoprotein lipase (LPL) hydrolizes TAG-rich lipoproteins, such as VLDL (89). LXR\(\alpha\)s also control the synthesis of LPL (90). This enzyme is located on the luminal surface of vascular endothelial cells. By hydrolyzing TAGs, it liberates FAs into adipose tissue for storage and into skeletal muscle for energy expenditure. Thus, LXR\(\alpha\)s are involved in FA metabolism by both promoting their synthesis and their clearance by uptake in peripheral tissues. As LXR\(\alpha\)s stimulate synthesis of FAs in the liver, the increased quantity of FAs in hepatocytes become available for synthesis of TAGs, which are subsequently secreted into the circulation as VLDLs. LXR\(\alpha\)^{-/-} mice exhibit disturbances in capacity to regulate genes involved in FA and TAG secretion due to deficiency of a number of lipogenic genes, such as the sterol regulatory element-binding protein (SREBP)-1c, FAS, acetyl-CoA carboxylase-\(\alpha\) (ACC-\(\alpha\)) and SCD-1 (30). FAS, ACC-\(\alpha\) and SCD-1 are directly regulated by SREBP-1c, supporting the role of LXR not only in sterol metabolism, but also in FA metabolism in general. LXR can also directly stimulate hepatic transcription of SREBP-1c (91;92), FAS (93) and ACC-\(\alpha\) (94) through LXREs in their promoters. Basal LXR activity is essential for hepatic SREBP-1c expression, further supporting the role of LXR in hepatic lipogenesis (95). Activation of both LXR and SREBP-1c seem to induce the lipogenic pathway to bigger extent compared to separate activation (93). Our group has previously investigated the link between LXRs and insulin, as the effect of LXR activation on hepatic gene expression is similar to the effects of insulin in this tissue. Tobin et al. reported that LXR\(\alpha\) expression in liver is induced by insulin (96). In addition, it seems that LXRs crosstalk with insulin to regulate hepatic lipogenesis, since insulin-mediated induction of key enzymes in hepatic FA synthesis were significantly suppressed in insulin-injected LXR\(\alpha^{-/-}\beta^{-/-}\) mice (96).

LXRs are also regulators of glucose metabolism. It has been previously shown that ligand activated LXRs inhibit expression of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting step in gluconeogenesis (97;98). This effect was paralleled by downregulation of 11\(\beta\)-hydroxysteroid dehydrogenase 1 (11\(\beta\)-HSD-1), which converts inactive corticosteroids into active corticosteroids (i.e. cortisol) (98). The downregulation of hepatic gluconeogenic genes, such as PEPCK and glucose-
phosphatase (G6P), in LXR agonist-treated mice, has also been shown by others (75;99). This inhibition was accompanied by induced glucokinase (GCK) expression, an enzyme which promotes hepatic glucose utilization (75). In addition, our group has shown that the insulin-dependent glucose transporter, GLUT4, is directly transactivated by LXR in adipose tissue (100). Along this line, activation of LXRs improves glucose tolerance in diabetic rodents through coordinate regulation of glucose metabolism in liver and adipose tissue (99). Another linkage between glucose and lipid metabolism mediated through LXR, was described by others when the expression of the carbohydrate responsive element binding protein (ChREBP), which promotes transcription of hepatic lipogenic enzymes, was shown to be regulated by LXR activation (101;102). This glucose-sensitive transcription factor is involved in converting excess carbohydrates to lipids (103;104).

Together, these data raise the intriguing issue of LXR as an important co-regulator of glucose and lipid metabolism, providing a molecular basis for the close relationship between these metabolic pathways (Figure 1.6.).
Figure 1.6. Convergence of glucose and lipid-sensing pathways on the nuclear receptor LXR. Mitro et al. (67) suggests that glucose is an endogenous ligand with the same efficacy to that of oxysterols, the known high-affinity ligand for LXR. The strength of LXR signaling can fluctuate widely, depending on nutritional states. LXR heterodimerise with RXRα in vivo and activate genes in the gluconeogenic/glycolytic, FA synthesis and cholesterol pathways in order to control nutrient and energy homeostasis. “?” denotes hypothetical or speculative elements. Modified from (105).

Synthetic LXR agonists inhibit atherosclerosis pathology in mice, an effect likely to result from modulation of both metabolic and inflammatory gene expression (106). Interestingly, LXR ligands could also mediate beneficial metabolic effects in insulin resistance syndromes, such as T2DM, by interfering with peripheral glucocorticoid activation. The antiatherogenic and antidiabetic effects observed in physiologic studies, is paralleled by a shift in metabolism to energy storage; a shift that includes lowering of glycemia and an increase in lipid accumulation (75). Furthermore, since the liver contains predominantly LXRα, LXRβ-specific agonists or tissue-specific LXR modulators may be effective in macrophage reverse cholesterol transport with less impact on LXRα mediated induction of hepatic lipogenesis. The design of LXR selective ligands is challenging due to the high homology in the LBDs in both LXR isoforms. Recently, Hu et al. were able to show that carboxylic acid based quinolines have some selectivity for LXRβ over LXRα in binding assays (107), but the search for selective LXR ligands is still under investigation.

1.3 The liver

The adult human liver weighs 1-1.5 kg, and lies under the diaphragm. It is supplied with blood from the hepatic artery (~20%) and the hepatic portal vein. This vein carries blood which has passed through the complex network of blood vessels surrounding the intestinal tract. Thus, the water-soluble substrates arising from the diet (i.e. monosaccharides and amino acids) are transported to the liver before entering the general circulation, giving the liver a crucial role in metabolism. A small group of veins, the pancreatic veins, join the portal vein before it enters the liver.
These veins carry blood containing insulin and glucagon from the endocrine part of the pancreas, allowing these hormones to exert their first effects on the liver, before being diluted in the general circulation. Almost half of the secreted insulin reaching the liver is removed in its “first passage.” Blood leaves the liver in numerous hepatic veins, which enters the inferior vena cava. Hepatic ducts carry bile acids to the gall bladder, located immediately under the liver (108). Bile contains bile salts, essential to digestion and absorption of fats from the intestine. During digestion, they reach duodenum through the common bile duct. Approximately 80% of the liver contains hepatocytes, arranged in hexagonal units (lobules). Other cell types include Kupffer cells and endothelial cells. The liver has important roles in carbohydrate metabolism (storage and release of glucose), amino acid metabolism and a smaller role in fat metabolism (28).

The liver can take up large amounts of glucose (i.e. following a carbohydrate-rich meal) and release it when it is required elsewhere in the body. Liver cells predominantly have GLUT2, a glucose transporter, which is not responsive to insulin and has a high $K_m$. Within hepatocytes, glucose is phosphorylated by GCK to glucose-6-phosphate. This glucose metabolite can enter the glycogenic pathway or be metabolised to pyruvate via glycolysis. In both pathways, the regulation is brought about by a change in the balance of hormones, including glucagon, catecholamines and insulin. In liver, the induction of genes encoding enzymes involved in de novo lipogenesis occurs in response to increased levels of glucose available (Figure 1.7). In this way, excess dietary carbohydrates can be converted into TAGs (109).

Like many other tissues, the liver is able to take up non-esterified fatty acids (NEFAs) from the plasma. These FAs can either be oxidized (mitochondrial $\beta$-oxidation) or used in TAG formation. Their fate is mainly controlled by insulin and glucagon. In fed state, when insulin is elevated, the liver tends to store FAs as TAG, rather than to oxidize them. The TAG formed by esterification of FAs, is stored within hepatocytes, appearing to be a local store for hepatic needs. The stored TAG acts as the substrate for hepatic secretion of fat into the bloodstream, in the form of VLDL. During
starvation or between meals, the ATP-required formation of glucose, the gluconeogenic pathway, can be fuelled by the oxidation of FAs. The liver also has a special role in cholesterol metabolism. Excess cholesterol is converted into bile acids and exported from the cell, while a simultaneously reduction in cholesterol biosynthesis and uptake of lipoprotein cholesterol takes place.

Figure 1.7. LXR regulate de novo lipogenesis in liver through target genes, directly (ACC, FAS, SCD-1) and indirectly (through SREBP-1c and ChREBP). Glucose is metabolized into Acetyl-CoA, which serves as a substrate for lipogenesis.
2. Objectives

The work in this thesis takes part in an EU funded project, CRESCENDO, with the overall aim to enhance the understanding of basic mechanisms underlying NR actions and their translation into the physiological regulation of development and the ageing process. CRESCENDO has 3 major aims:

- To enhance understanding of NR signaling in the context of regulatory networks.

- To study the roles of NRs in the continuum of development to ageing, with emphasis on the complex links between NR signaling, metabolic disease, as well as brain development and ageing.

- To transfer this knowledge into therapeutic leads.

In this master thesis I want to investigate the link between the ageing process and how it affects the glucose and lipid metabolism \textit{in vivo}. The main goal is to elucidate the contribution of LXR\(\alpha\) and its interacting ascending and descending signaling cascades in regulating glucose and lipid homeostasis in ageing under normal physiological conditions. In order to contribute and achieve this goal, we included female and male LXR\(\alpha^{--}\) and WT mice (1, 3, 6 and 9 months old).

The objectives of this thesis are to:

- Describe changes in ageing WT and LXR\(\alpha^{--}\) mice (female and male, 1-9 months) regarding:

  o Bodyweight, organweight (liver, white adipose tissue (WAT))

  o Physiological parameters in serum (glucose, insulin, cholesterol, FAs, TAGs)
Hepatic gene expression encoding proteins in glucose, lipid and cholesterol metabolism, as well hepatic genes encoding TFs (SREBP-1, ChREBP) and NRs (ERα). In order to analyze the transcriptional cascades in the liver, hepatic total RNA is isolated by Trizol® reagent, cDNA is synthesized and qRT-PCR is applied.

- Describe differences (bodyweight, organweight, serum levels, and hepatic gene expression) between male and female mice with increased lifespan (1-9 months).
### 3. Materials

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>Sigma</td>
</tr>
<tr>
<td>DEPC</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Merck</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>Arcus</td>
</tr>
<tr>
<td>RNase-away</td>
<td>Molecular Bio Product</td>
</tr>
<tr>
<td>Taqman® Universal Master Mix</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>TRIZOL® Reagent</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Falcontubes</td>
<td>Falcon</td>
</tr>
<tr>
<td>MicroAmp Optical 96-well Reaction Plate</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Microtubes</td>
<td>Axygen</td>
</tr>
<tr>
<td>PCR-tubes</td>
<td>Sarstedt</td>
</tr>
<tr>
<td>Pipettes</td>
<td>Biohit</td>
</tr>
<tr>
<td>Repeating pipettes</td>
<td>Rainin</td>
</tr>
<tr>
<td>Pipette-tips</td>
<td>Thermo</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Instruments</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accu chek Aviva (Glucometer)</td>
<td>Roche</td>
</tr>
<tr>
<td>Acculab Econ (Weight)</td>
<td>Acculab</td>
</tr>
<tr>
<td>AX105 DeltaRange (Weight)</td>
<td>Mettler Toledo</td>
</tr>
<tr>
<td>ABI PRISM® 7900HT SDS</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Biofuge fresco (Centrifuge)</td>
<td>Heraeus instruments</td>
</tr>
<tr>
<td>Titertek Multiskan ® PLUS</td>
<td>Labsystems Oy</td>
</tr>
<tr>
<td>Freezer, -76 °C</td>
<td>Forma Scientific</td>
</tr>
<tr>
<td>Equipment/Software</td>
<td>Manufacturer</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>GeneAmp PCR System 9700</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>ND-1000 Spectrophotometer</td>
<td>NanoDrop</td>
</tr>
<tr>
<td>MilliQ-synthesis</td>
<td>Millipore</td>
</tr>
<tr>
<td>Pipeteboy Comfort</td>
<td>Integra Biosciences</td>
</tr>
<tr>
<td><strong>Kits</strong></td>
<td><strong>Manufacturer</strong></td>
</tr>
<tr>
<td>High Capacity cDNA Archive Kit</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>TaqMan Gene Expression Assay</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Low Density Arrays</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Ultrasensitive Mouse Insulin ELISA</td>
<td>Mercodia</td>
</tr>
<tr>
<td><strong>Software and internet resources</strong></td>
<td><strong>Manufacturer</strong></td>
</tr>
<tr>
<td>Adobe Illustrator CS2/CS3</td>
<td>Adobe</td>
</tr>
<tr>
<td>Primerdesign</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Microsoft Office</td>
<td>Microsoft</td>
</tr>
<tr>
<td>NanoDrop Software V.3.2.1</td>
<td>Nanodrop</td>
</tr>
<tr>
<td>Pubmed</td>
<td>National Library of Medicine</td>
</tr>
<tr>
<td>RQ Manager Software</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Sequence Detection System software</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>SPSS 14.0 for Windows</td>
<td>SPSS Inc.</td>
</tr>
</tbody>
</table>
4. Methods

4.1 Animal experiments

All use of animals have been approved and registered by the Norwegian Animal Research authority. Animals used in this experiment were LXRα⁻/⁻β⁺/+ mice, generated as previously described (110-112) as well as wild type littermates. All mice had a similar mixed genetic background based on 129/Sv and C57BL/6 strains, backcrossed in C57BL/6J mice for ten generations. The animals were housed in a controlled environment; constant temperature (22°C) and humidity, light from 6 a.m. to 6 p.m. They were fed ad libitum on a standard chow diet with the following composition in terms of energy: 64 % carbohydrate, 4.5 % lipids and 31.5 % proteins (Special Diet Services). Animals had free access to water throughout the experiment. They were housed according to age, genotype and sex, four to six animals per cage. Upon arrival the mice were acclimatized for at least one week before sacrifice. At the time of sacrifice, body weight was measured, correct to ± 0.1 g using an Acculab ECON scale. All animals were euthanized by cervical dislocation at the same time of the day, three hours after the end of dark cycle (Zeitgeber Time 3, ZT3) at 1, 3, 6 or 9 months of age. Blood was collected by cardiac puncture, glucose was measured and organs (heart, liver, pancreas, white adipose tissue [epididymal and subcutaneous], musculus soleus and musculus gastrocnemius) were excised. The weight of liver and epididymal adipose tissue was registered, and a small piece was taken to determine the lipid profile in the tissues. Organs were snap frozen in liquid nitrogen (-196°C) and stored at -76°C until isolation of total RNA. Separation of serum was done by centrifuging the blood samples at 7000 rpm, 4°C for 7 min. Serum was transferred to new tubes and stored at -76°C until further analyzes.
4.2 RNA analysis

RNA is at risk of degradation by ribonucleases (RNases), which may be introduced accidentally into the RNA preparation at any point in the isolation process through improper technique. Contamination may come from the lysed cells, working solutions or most commonly; bacteria and molds on the hands of the worker or dusty laboratory equipment. In the presence of TRIzol® reagent, RNA is protected from RNase contamination. Downstream sample handling requires that nondisposable glassware or plasticware is RNase-free. RNase activity is difficult to inhibit. It is therefore important to prevent its introduction by proper microbiological, aseptic technique:

- All the equipment used is sterile or washed with RNase Away followed by rinsing with RNase free water before being used.

- All water used is Diethyl-pyro-carbonate (DEPC) water (see 4.2.1) or PCR-grade water.

- All handling is done using disposable gloves to prevent RNase contamination from surface of the skin. Gloves should be changed frequently.

- Keep tubes closed whenever possible.

- Equipment and working solutions are kept sterile.

- Tissues are stored at -76°C, and transported in liquid nitrogen.

RNase can be deactivated by heat inactivation at 150°C for four hours. Isolated RNA is always kept on ice; RNA is stored at -76°C.

Isolated total RNA can be used for a number of down-stream applications like; Northern blot analysis, dot blot hybridization, poly (A)+ selection, in vitro translation, RNase protection assay, molecular cloning or cDNA-synthesis.
4.2.1 DEPC-water

DEPC is a strong, but not absolute, inhibitor of RNases. DEPC inactivates RNases by covalently modification, and is used to create RNase-free water. This is done by adding 125 μl DEPC to 1 l Milli-Q water (0.0125%), which is deionized water that has been purified in a Milli-Q system. The solution is then mixed and incubated at 37°C for 12 hours to remove the RNase present. Residual DEPC is autoclaved to eliminate the DEPC.

4.2.2 Isolation of total RNA from liver tissue

TRIzol® reagent, purchased from Invitrogen (Cat.No 15596-018), was used to isolate total RNA from liver tissue. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (113). Guanidine isothiocyanate and chloride denature proteins and inhibit RNases. RNA integrity is maintained while the cell structure is ruptured by denaturation of proteins, and nuclear proteins dissociates from the nuclear acids. Addition of chloroform, followed by centrifugation, provides a phenol-chloroform extraction of RNA and separates the solution into a colorless upper aqueous phase, an interphase and a lower red, phenol chloroform phase. RNA remains exclusively in the upper aqueous phase. After transferring the aqueous phase, RNA can be precipitated with isopropyl alcohol. Total RNA isolated by this method is free of protein and DNA contamination.

Protocol

Prior to homogenization, keep the tissue in liquid nitrogen. Homogenize 50 to 100 mg tissue using an Ultra Turrax® T8 (IKA LABORTECHNIK, Staufen Germany) in 1 ml TRIzol® for 20-30 sec. Incubate homogenized samples for 5 min at 15-30°C to allow the complete dissociation of nucleoprotein complexes. Add 0.2 ml chloroform per 1 ml of TRIzol® used for homogenization. Cap sample tubes securely. Shake tubes vigorously by hand for 15 sec, and incubate at room temperature for 2-3 min.
Centrifuge the samples at no more than 12,000 × g for 15 min, 4°C. Transfer the RNA, which remains exclusively in the upper aqueous phase following centrifugation, to a fresh tube. Precipitate the RNA from the aqueous face by mixing with isopropyl alcohol. Use 0.5 ml isopropyl alcohol per 1 ml of TRIzol® reagent used for the initial homogenization. Incubate samples at room temperature for 10 min and centrifuge at no more than 12,000 × g for 10 min at 4°C. RNA is now visible as a gel like pellet on the side and bottom of the tube. Remove the supernatant. Wash with 75% ethanol, vortex and centrifuge at no more than 7,500 × g for 5 min, 4°C. Remove ethanol subsequently and briefly air-dry (not completely) the pellet for 5-10 min. Re-dissolve the pellet in DEPC-H₂O and incubate at 55°C for 10 min. Isolation is now completed and RNA should be stored at -76°C.

Spectrophotometric quantification of nucleic acids
Concentration, quality and purity of the isolated RNA were determined using a NanoDrop® ND-1000 Spectrophotometer that accurately and reproducibly measures 2 µl nucleic acid samples. It has high absorbance capacity and can measure RNA-concentrations in the range of 2.0-3000 ng/µl. To do this, the instrument automatically detects the absorbance and utilizes the 0.2 mm light pathlength to calculate the concentration. Nucleic acids absorb light in the ultraviolet range (200-400 nm) with an absorption peak at 260 nm. Proteins have an absorption peak at 280 nm. The ratio OD260/OD280 provides an estimate of purity of the DNA and RNA. A ratio of ~ 2.0 is generally accepted as “pure” for RNA. An appreciably lower ratio may indicate presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. All samples used had a ratio higher than 1.8. The ratio OD260/OD230 is a secondary measure of nucleic acid purity. This value is commonly higher (1.8-2.2) than the OD260/OD280 ratio. A very low value may indicate the presence of co-purified contaminants.
4.2.3 cDNA synthesis

High Capacity cDNA Archive Kit (P/N: 4322171, Applied Biosystems) was used to reverse transcribe total RNA to single-stranded complimentary DNA (cDNA). A poly-A sequence at the 3’ end of mRNA is used as a template. The kit uses the random primers scheme for initiating cDNA synthesis. Random primers ensure that the first strand synthesis occurs efficiently with all species of RNA present, including mRNA and rRNA. An essential requirement for the relative quantification of cDNA is that the reverse transcription (RT) reaction generates products in a manner directly dependent on the amount of input RNA template. The first strand of cDNA can be directly used as a template in the polymerase chain reaction (PCR). Conversion of total RNA to cDNA is also preferred, as cDNA is considered to be more stable for long term archiving and is less susceptible for degradation than RNA.

Protocol
Preparing RNA samples and 2×RT master mix

The reaction volume in the cDNA synthesis was set to 50 µl (25 µl RNA sample and 25 µl master mix). In this work an input of 3.0 µg total- RNA was used. Before sample preparation, allow samples and kit components to thaw on ice. Then prepare 3.0 µg RNA samples in sterile, RNase free tubes and adjust volume to 25 µl with PCR grade water, mix and spin down. The kit contains reagents, which when provided, forms a 2×RT master mix. As such, an equal volume of both RNA sample and 2×RT master mix should be added. Prepare 2×RT master mix. Pipette 25 µl 2×RT master mix to each well on a RNase-free 96 well reaction plate. Then pipette 25 µl RNA sample into wells, pipetting up and down two times to mix. Seal the plate with caps, vortex and briefly spin down to eliminate any air bubbles. Place the plate on ice until you are ready to load the thermal cycler (GeneAMP 9700). Incubate at 25°C for 10 min and 37°C for two hours. By completion of the reaction the converted cDNA is held at 4°C for short term storage (up to 24 hours). For long time storage and archiving, store at either -20°C or -76°C.
Preparing a 2×RT master mix (20 reactions): 210 µl RNase-free H2O, 100 µl 10× Random Primers, 100 µl 10×RT Buffer, 40 µl 25× dNTP mixture and 50 µl Multiscribe RT (50 U/ µl). Place the 2×RT master mix on ice until the cDNA reaction plate is prepared.

4.3 Essential RNA - DNA techniques

4.3.1 Real time polymerase chain reaction

Real Time PCR
The PCR is an enzymatic in vitro amplification of a specific DNA segment. DNA polymerase amplifies target cDNA synthesized from total RNA, using two sequence-specific, unlabelled primers and a TaqMan MGB probe (6-FAM dye-labelled) from the TaqMan Gene Expression Assay mix. Real Time PCR, also known as qPCR, qRT-PCR, RT-qPCR and kinetic PCR, is a quantitative PCR method to determinate the copy number of PCR templates, such as DNA or cDNA, in a PCR reaction. Reactions are characterized by the point in time during cycling when amplification of a target is first detected above a certain threshold, rather than the amount of target accumulation after a fixed number of cycles. The higher the copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. There are two types of real time PCR: probe based (TaqMan PCR) and intercalator based (SYBR Green PCR). Both methods require a special thermocycler equipped with a sensitive camera that detects fluorescence in each well of a 96-well plate at frequent intervals during the PCR reaction. Unlike the SYBR Green method, which uses a fluorescent dye that only binds to double-stranded DNA, TaqMan PCR uses a fluorogenic probe designed to bind only the DNA sequence between the two PCR primers. The fluorogenic probe is a single stranded oligonucleotide of 20-26 nucleotides that contains a reporter dye and a quencher dye, covalently attached to its 5′ and 3′ ends, respectively. When the probe is intact, the proximity of the reporter dye to the quencher dye, results in suppression of the reporter fluorescence. During
PCR, if the target of interest is present, the probe specifically anneals to a complimentary sequence between the forward and reverse primer sites. The 5'-3' nucleolytic activity of the DNA polymerase system only cleaves probes hybridized to the target (Figure 4.1). Cleavage of the probe separates the reporter dye and the quencher dye, displacing probe fragments from the target, while polymerization of the strand continues. This separation results in increased fluorescence of the reporter.

![Diagram of PCR process](image)

**Figure 4.1. 5’-3’Nuclease activity of the DNA polymerase system (114).**

The 3’ end of the probe is blocked, preventing extension of the probe during the reaction. This process occurs in each cycle and does not interfere with the exponential accumulation of the product. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter. The increase in fluorescence is generated only if the target sequence is complimentary to the probe
and is amplified during PCR. Therefore any nonspecific amplicons are not detected in TaqMan PCR, making it more accurate and reliable than the SYBR Green method. Nevertheless, special laboratory practices are required to prevent contamination and avoid false positive amplifications (115). The high sensitivity of the PCR assays can lead to amplification of a single DNA molecule (116). Follow general PCR practices as used when handling RNA. In addition:

- Maintain separate areas, dedicated equipment and supplies for sample preparation, PCR setup, PCR amplification and analysis of PCR products.
- All surfaces in the PCR area should be routinely decontaminated to prevent cross contamination.
- Never bring amplified PCR products into the PCR setup area.
- Keep reactions and components capped as much as possible.
- Open and close all reaction plates and sample tubes carefully. Try not to splash or spray PCR samples.

Besides reagents required for normal PCR, TaqMan PCR requires the abovementioned PCR primers with a preferred product size of 50-150 bp, a fluorogenic probe and a real-time PCR machine.

Before PCR starts, a hold at 95ºC is needed to activate the heat stable DNA polymerase. The PCR consists of three steps. Firstly the denaturation of the DNA at 95ºC, secondly annealing at 60ºC, where primers bind to the single stranded DNA, and thirdly the elongation of primers, catalysed by DNA polymerase, still at 60ºC, the optimal temperature for the heat stable DNA polymerase used. This cycle is repeated 40-45 times. The amount of target product increases exponentially. If a maximum efficiency is obtained, this increase is given by the equation $N = N_0 \times 2^n$, where $N$ equals the number of amplified fragments, $N_0$ equals the initial number of fragments and $n$ equals the number of amplification cycles. In the system used in this work maximal efficiency can be assumed.
Primer design

A pair of primers is needed by DNA polymerase to amplify the DNA target (i.e. a specific genetic sequence) of interest. TaqMan® Gene Expression Assays, ordered from Applied Biosystem (www.appliedbiosystems.com), are a comprehensive collection of predesigned gene-specific primer and probe sets. TaqMan Gene Expression Assays are built on 5’ nuclease chemistry. Each assay consists of two unlabeled PCR primers and a FAM dyelabeled TaqMan® MGB (minor groove binder) probe. All components are quality control-tested and formulated as a single 20 × mix. They are designed to run under universal conditions for two-step RT-PCR and amplify target cDNA without amplifying genomic DNA (m suffix in assay ID), when possible. This is achieved by designing probes that cross exon-exon junctions.

Primers for specific endogenous controls must be carefully selected. Ideally, an endogenous control should be constitutively expressed in all cells, preferably expressed in similar levels as the gene(s) of interest and its expression should not change in the application. In general, housekeeping genes (i.e. 18S, 36B4, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) etc.) are the typical choice due to their mostly consistent expression levels in all cell types. The selected endogenous reference should be determined empirically and based on the system under study.

Protocol (96 well format)

The PCR step must be performed on a Real-Time PCR System. Traditional thermal cyclers cannot be used, because they cannot detect and record the fluorescent signals generated by the cleavage of the TaqMan probes. Perform the PCR in a 96 well format. Use 10-100 ng of cDNA per 20 µl reaction volume. Despite the specificity of Real-Time PCR using the TaqMan Gene Expression Assay, no template control (NTC) and “no amplification control” (without reverse transcriptase) should be run in order to check for genomic DNA contamination etc.

REAGENT PREPARATION: Thaw any frozen cDNA samples and TaqMan Gene Expression Assay mix (light sensitive) on ice. When thawed, resuspend by vortexing
and then centrifuge the tubes briefly. Prior to use, mix the PCR master mix thoroughly by swirling the bottle. Prepare the PCR reaction mix for each sample. Mix the solutions by gently pipetting up and down. Cap the tubes and centrifuge them briefly.

PCR REACTION PLATE PREPARATION: Each 20 µl reaction consists of: 10 µl TaqMan Universal PCR Master Mix (2×) No AmpErase UNG (P.N: 4324018), 8 µl PCR grade H2O and 1µl TaqMan Gene Expression Assays (20×) and 1 µl cDNA template. Transfer the correct volume of each reaction mixture to wells of a reaction plate that can be used on the 7900HT system. Cover the plate with an optical adhesive cover. Centrifuge the plate briefly to spin down content and eliminate any air bubbles from the solution that may be present. Visually verify that each reaction is positioned at the bottom of its well. Apply a standard compression pad to the sealed optical plate.

RUNNING THE PLATE: Place the reaction plate in the instrument. Run the plate on the 7900HT system and select the standard run.

ANALYZING RESULTS: This involves three procedures:

1. View the amplification plots for the entire plate to ensure that the amplification has been successful.

2. Set the baseline and threshold values.

3. Calculate relative gene expression levels. Results were obtained from the 7900HT system using the Comparative CT method for relative quantification. A CT (cycle threshold) value is the fractional cycle number at which the fluorescence passes the fixed threshold. The threshold should be set in region associated with an exponential growth of the PCR product. To minimize the variation in input cDNA, signals generated by the amplifications of the target sequence in the cDNA samples should be normalised to signals obtained from endogenous controls. For the comparative CT method to be valid, the
efficiency of the target amplification (the gene of interest) and the reference amplification (the endogenous control) must be approximately equal. The formula $2^{-\Delta\Delta CT}$ was used to calculate results.

**Low Density Array (LDA)**

LDA cards (Figure 4.2) were also used to profile gene expression. These cards permit the amplification of endogenous controls and targets in cDNA samples using fluorogenic 5’ nuclease assays. The LDA card functions as an array of reaction vessels for the PCR/sequence detection step. It consists of 8 series of 48 intercalated wells. Each set provides a user-specified number of replicates. The wells contain dried Applied Biosystems TaqMan probes, labelled with the FAM dye, and primers for one mRNA target. Relative levels of gene expression were determined from the fluorescence data generated during the PCR using the ABI PRISM 7900HT Sequence Detection System and Comparative CT method using the Relative Quantification software.

![Figure 4.2: The Low Density Array Card. Adapted from (114).](image)

**Protocol Low Density Array**

Do not remove the LDA card from its packaging before it has reached room temperature and you are ready to load it with sample-specific PCR reaction mix.
Prolonged exposure to indoor lightning can photo-degrade the fluorescent probes contained within the micro fluidic card.

**PREPARE THE SAMPLE-SPECIFIC PCR MIX:** For each sample, add the following components to a labelled 1.5 ml microcentrifuge tube: 5 µl cDNA sample, 45 µl RNase/DNase-free water, 50 µl TaqMan Universal PCR master mix (2×). Cap the tube. Mix thoroughly by gentle vortexing and centrifuge briefly.

**LOAD FILL RESERVOIRS:** Load 100 µl of the desired sample-specific PCR reaction mix, made from a single cDNA sample, into a 100 µl micropipette and dispense the entire volume in the fill port reservoir of the LDA card (foil side down). Insert the LDA card into a card holder and centrifuge the card to transfer the sample-specific PCR reaction mix from the fill reservoirs to the reaction wells. Two consecutive 1 min centrifugations at 1200 rpm are needed to ensure complete distribution of the sample-specific PCR reaction mix and resuspend the dried Taqman probes and primers within the wells of LDA card. After centrifugation, examine each LDA card to determine whether the filling is complete.

**SEAL THE LDA CARDS:** Use a LDA card sealer. Sealing should be done as soon as possible following centrifugation, reducing the risk of cross-contamination. If the sealing is successful, the LDA card is ready to be run on the 7900HT system.

### 4.4 Serum analysis

When handling serum samples; minimize freeze/thaw cycles, time that serum is thawed and sample temperature by keeping samples on ice or at 4ºC.

#### 4.4.1 Glucose Quantification

The measurement of glucose in blood was performed immediately after blood was taken out by using an Accu-Chek Aviva. A droplet of blood was loaded on the front
yellow part of a test strip, already inserted into the Accu-Chek Aviva. The instrument automatically calculates the plasma glucose value.

### 4.4.2 Insulin Quantification

An ELISA (enzyme-linked immunosorbent assay) allows for rapid screening and quantification of the presence of an antigen in a sample. Mercodia Ultrasensitive Mouse Insulin ELISA kit (Cat.No: 10-1150-01) is a solid two-site immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are targeted against different antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microtitration well. A simple washing step removes unbound material in serum and unbound enzyme labelled antibody. The bound conjugate is detected by reaction with 3.3′,5.5′ tetramethylbenzidine (TMB). Adding acid stops the reaction and gives a colorimetric endpoint. The enzyme activity can be measured spectrophotometrically by the increased absorbance at 450 nm. The increase in absorbance is directly proportional to the amount of captured insulin in the unknown samples. The concentration of insulin can be calculated by interpolation from a calibration curve generated in the same assay with reference standards of known concentrations of mouse insulin.

### Protocol

Store the kit components at 2 - 8°C. Each kit contains reagents for 96 wells, sufficient for 42 samples and one Calibrator curve in duplicate. Thaw serum samples on ice, vortex and centrifuge briefly. Put all kit reagents and samples to room temperature before use. Perform each determination in duplicates for calibrators and unknowns. Prepare a calibration curve for each assay run. Add 25 µl of calibrator 0 to each anti-insulin well. Calibrators 3 to 7 and serum samples are performed in duplicates, 5 µl in each well. Add 50 µl enzyme conjugate to each well. Avoid contamination between the conjugate and substrate, by separating pipettes. Incubate on a plate shaker (700-900 cpm, orbital movement) for 2 hours at room temperature. Aspirate the reaction
volume and wash six times with 350 µl Wash Buffer. After final wash, invert and tap
the plate firmly against absorbent paper. Add 200 µl Substrate TMB. Cover the plate
in aluminum foil and incubate for 30 min. Add 50 µl Stop Solution, shake the plate
gently for 5 sec to ensure mixing of Substrate and Stop Solution. Measure the
absorbance at 450 nm.

Solutions
Calibrators: Add 1 ml of redistilled water to each of the six calibrators. Calibrator 0
is ready for use.

Enzyme conjugate: Gently mix 600 µl of Enzyme Conjugate 11× with 6 ml of
enzyme conjugate buffer.

Substrate TMB: Ready for use. Light sensitive!

Wash buffer: Dilute the wash buffer (40 ml) 21× in redistilled water (800 ml).

Stop solution: Ready for use.

4.4.3 Lipid Quantification

Serum lipid quantification, using enzymatic in vitro tests, was performed by others on
a Roche Hitachi 917. NEFAs were determined with a Wako NEFA C-kit (994-
75409), TAGs with a Roche Triglyceride GPO-PAP kit (11730711216) and
cholesterol with a Roche Cholesterol CHOD-PAP kit (11491458216).

4.5 Statistics

All statistics were calculated using SPSS Software 14.0. The results are presented as
mean of n values ± SEM. The level of statistical significance was set at P < 0.05.
Significant differences were established by the Mann-Whitney test. General
differences between groups (WT females, WT males, LXRα−/− females or LXRα−/−
males) were tested by pooling all data from 1-9 months within each group. All correlation analysis was compared by Spearman correlation coefficient.
5. Results

5.1 Animal characteristics in female and male WT and LXRα⁻/⁻ mice, aged 1-9 months

C57BL/6 female and male mice (LXRα⁻/⁻ and WT) were aged for 1, 3, 6 or 9 months. The bodyweight and organweight (liver and WAT) increase significantly from 1-9 months in all mice (Table 5.1).

<table>
<thead>
<tr>
<th>Wild Type</th>
<th>Sex</th>
<th>1 month</th>
<th>3 months</th>
<th>6 months</th>
<th>9 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>M</td>
<td>22.6 ± 0.7</td>
<td>31.0 ± 1.0 *</td>
<td>35.3 ± 1.2 *</td>
<td>41.7 ± 2.9 *</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>20.2 ± 0.6</td>
<td>21.2 ± 0.5</td>
<td>30.0 ± 1.3 *</td>
<td>29.8 ± 0.9 *</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>M</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.8 ± 0.1 *</td>
<td>1.9 ± 0.1 *</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.0</td>
<td>1.4 ± 0.1 *</td>
<td>1.2 ± 0.1 *</td>
</tr>
<tr>
<td>WAT (g)</td>
<td>M</td>
<td>0.3 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.6 ± 0.1 *</td>
<td>1.2 ± 0.1 *</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.6 ± 0.1 *</td>
<td>0.6 ± 0.1 *</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>M</td>
<td>11.6 ± 0.6</td>
<td>9.9 ± 0.4 *</td>
<td>10.1 ± 0.7</td>
<td>9.3 ± 1.6 *</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>7.9 ± 1.2</td>
<td>8.3 ± 0.5</td>
<td>6.7 ± 0.5</td>
<td>8.5 ± 0.6</td>
</tr>
<tr>
<td>Insulin (µg/l)</td>
<td>M</td>
<td>0.7 ± 0.0</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>1.7 ± 0.4 *</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.8 ± 0.1</td>
<td>0.5 ± 0.0 *</td>
<td>1.1 ± 0.2</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>M</td>
<td>2.2 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>2.1 ± 0.2</td>
<td>1.9 ± 0.0</td>
<td>2.1 ± 0.1</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>TAG (mmol/l)</td>
<td>M</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.8 ± 0.2 *</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.0</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>FFA (mmol/l)</td>
<td>M</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LXRα⁻/⁻</th>
<th>Sex</th>
<th>1 month</th>
<th>3 months</th>
<th>6 months</th>
<th>9 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>M</td>
<td>21.2 ± 1.1</td>
<td>34.6 ± 0.3 *</td>
<td>37.4 ± 1.1 *</td>
<td>39.9 ± 0.8 *</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>16.6 ± 0.5</td>
<td>22.7 ± 0.4 *</td>
<td>24.0 ± 0.8 *</td>
<td>31.5 ± 2.3 *</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>M</td>
<td>1.2 ± 0.0</td>
<td>1.8 ± 0.1 *</td>
<td>1.9 ± 0.0</td>
<td>1.8 ± 0.1 *</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.8 ± 0.0</td>
<td>1.0 ± 0.0 *</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.1 *</td>
</tr>
<tr>
<td>WAT (g)</td>
<td>M</td>
<td>0.1 ± 0.0</td>
<td>0.3 ± 0.1 *</td>
<td>0.7 ± 0.2 *</td>
<td>0.9 ± 0.1 *</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>&lt;0.0 ± 0.0</td>
<td>0.2 ± 0.9 *</td>
<td>0.3 ± 0.1 *</td>
<td>0.5 ± 0.1 *</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>M</td>
<td>10.7 ± 0.7</td>
<td>11.6 ± 1.1</td>
<td>8.6 ± 1.0</td>
<td>9.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>7.9 ± 0.2</td>
<td>7.3 ± 0.9</td>
<td>7.3 ± 0.7</td>
<td>9.0 ± 1.0</td>
</tr>
<tr>
<td>Insulin (µg/l)</td>
<td>M</td>
<td>0.9 ± 0.1</td>
<td>1.8 ± 0.3 *</td>
<td>1.8 ± 0.1 *</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.7 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>1.1 ± 0.2 *</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>M</td>
<td>2.5 ± 0.1</td>
<td>2.1 ± 0.1 *</td>
<td>2.1 ± 0.2</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1.8 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>TAG (mmol/l)</td>
<td>M</td>
<td>1.4 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.0</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>FFA (mmol/l)</td>
<td>M</td>
<td>1.7 ± 0.1</td>
<td>0.8 ± 0.2 *</td>
<td>0.9 ± 0.1 *</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1.5 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>0.8 ± 0.1 *</td>
</tr>
</tbody>
</table>
Table 5.1. Animal characteristics in ageing WT and LXRα−/− mice. (A) WT and (B) LXRα−/− male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had ad libitum access to water and food (standard chow diet). N=4 in each group. Blood was taken from the heart and stored on ice until separation of serum. Serum analysis was performed as described in materials and methods. Values are means ± SEM. *p<0.05 vs 1 month values.

Female mice, both LXRα−/− and WT, are significantly smaller than male mice, but no significant genotype-related differences are found regarding bodyweight and organweight. In addition, 9 month old LXRα−/− and WT males weigh more or less 40 gram, which is generally considered as obese.

Furthermore, we measured glucose in blood (values are plasma calibrated) and different parameters in serum, including insulin and lipids (FFAs, cholesterol, TAG). The serum concentrations of TAGs, cholesterol, FFAs are not altered in an age-dependent fashion in the mice studied (1-9 months) (Table 5.1). However, the levels of TAGs and cholesterol, but not FFAs are significantly lower in female mice of both genotypes. Albeit not significant (P = 0.056), LXRα−/− mice exhibit lower levels of cholesterol and TAG in serum compared to their WT littermates.

The plasma glucose levels fluctuate within each group from 1-9 months, but are significantly higher in male mice of both genotypes compared to female animals. Insulin levels are also significantly higher in male mice compared to female mice, in both genotypes (Table 5.1 and Figure 5.1). In contrast to the glucose concentration, serum insulin levels are significantly age-induced in male WT mice (1- 9 months). The insulin levels are also significantly induced in male LXRα−/− mice, but this induction is only significant when comparing 1 to 3 months, and 1 to 6 months. In female LXRα−/− mice, there is a small, significant induction between 1 and 9 months, but this is not observed in female WT mice.
Figure 5.1. Insulin levels in serum of WT and LXRα−/− (KO) male (M) and female (F) C57BL/6 mice, 1-9 months of age. Animals were housed in a controlled environment and had ad libitum access to water and food (standard chow diet). N=4 in each group. Blood was taken from the heart and stored on ice until separation of serum. Serum analysis was performed as described in the text. Values are means ± SEM. *p<0.05 vs 1 month values.

Given that males weigh more than females, we thought it would be interesting to study the correlation between bodyweight and serum levels. Insulin levels correlate significantly with bodyweight and organweight (liver, WAT), whereas other measured serum components do not show similar correlation (1-9 months). Nevertheless serum TAG levels correlate significantly with serum levels of cholesterol and FFAs (1-9 months).

5.2 Hepatic expression of selected genes in ageing mice (1-9 months)

LXRs have profound effects in glucose, lipid and cholesterol metabolism. As LXRα is the dominant LXR isoform in liver (85), we investigate how LXRα influences the glucose and lipid metabolic pathways during ageing (1-9 months) in the liver of female and male LXRα−/− mice compared to WT littermates. This is done by
analyzing some basal mRNA expressions of genes involved in regulatory pathways, both ascending and descending, in glucose and lipid homeostasis. In addition, sex-related changes are investigated, as discussed in section 5.2.5.

The inhibitor of cyclin-dependent kinase 4 (INK-4) gene is a tumour suppressor gene. Given that the expression of INK-4 is known to increase during ageing in mammals (117), we selected INK-4 as a positive control for the ageing of mice in this study. With the exception of WT males, hepatic INK4 mRNAs increase significantly from 1-9 months in the mice studied (Figure 5.2).

Figure 5.2. Relative mRNA levels encoding INK4 in vivo. WT and LXRα−/− (KO) male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had ad libitum access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol® reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. #p<0.05 1 month vs 9 month values.

5.2.1 Genes involved in hepatic glucose metabolism

Investigated genes at the mRNA level involved in glucose metabolism, include genes involved in glucose transport (GLUT2), glycolysis (GCK [phosphorlylates glucose to glucose 6-phosphate] and gluconeogenesis (PEPCK [decarboxylates oxaloacetate to
phosphoenolpyruvate], FBP-1 [dephosphorylates fructose 1,6-bisphosphate], G6P [hydrolyses glucose 6-phosphate to free glucose]) (26). None of these genes have identified LXRE(s) in their promoter.

Regarding glucose transport, it is known that GLUT4 is a direct target gene of LXRα in adipose tissue (100). However, the hepatic GLUT2 is not a known LXRα target gene. As such, we thought it would be interesting to investigate if LXRα regulates GLUT2 in liver. Indeed, the hepatic basal GLUT2 mRNA level is significantly lower in 1 month old female and male LXRα−/− mice compared to their WT littermates (Figure 5.3). Furthermore, GLUT2 mRNAs are significantly age-induced in LXRα−/− male mice from 1-9 months. In 9 month old LXRα−/− male mice the basal expression of GLUT2 is significantly higher compared to the mRNA levels in 9 month old WT males. A similar, albeit not significant pattern is observed in female LXRα−/− mice.

Figure 5.3. Relative mRNA levels encoding GLUT2, involved in hepatic glucose transport in vivo. WT and LXRα−/− (KO) male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had ad libitum access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol® reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. *p<0.05 WT 1 month vs LXRα−/− 1 month values. #p<0.05 1 month vs 9 month values.
The hepatic basal mRNA expression of GCK, involved in glycolysis, is only significantly higher in 1 month old female LXRα−/− mice compared to their WT controls. However, GCK is significantly age-induced at mRNA level from 1 to 9 months in female and male LXRα−/− mice (Figure 5.4). A similar trend is observed in WT mice, but this is not significant.

Figure 5.4. Relative mRNA levels encoding GCK, involved in glycolysis in vivo. WT and LXRα−/− (KO) male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had ad libitum access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol® reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. *p<0.05 WT 1 month vs LXRα−/− 1 month values. #p<0.05 1 month vs 9 month values.

The basal mRNA expression of selected gluconeogenic genes (PEPCK, FBP-1 and G6P) are significantly downregulated in 1 month old male LXRα−/− mice compared to WT males (Figure 5.5). In 1 month old female LXRα−/− mice, this downregulation is only significant for the PEPCK gene. Furthermore, PEPCK mRNA levels increase significantly from 1-9 months in LXRα−/− male mice. A similar, but not significant trend is observed in LXRα−/− female mice. Interestingly, in WT mice the mRNA levels of the PEPCK gene seem to decrease from 1-9 months, however only significant in females. We also demonstrate a significantly higher expression of G6P mRNAs
between 1 and 9 months in LXRα−/− female mice. Nevertheless, G6P mRNA levels seem to fluctuate within this group and do not show a clear age-dependent trend.

Figure 5.5. Relative mRNA levels encoding genes involved in gluconeogenesis in vivo. WT and LXRα−/− (KO) male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had ad libitum access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol® reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. *p<0.05 WT 1 month vs LXRα−/− 1 month values. #p<0.05 1 month vs 9 month values.

Figure 5.5. Relative mRNA levels encoding genes involved in gluconeogenesis in vivo. WT and LXRα−/− (KO) male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had ad libitum access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol® reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. *p<0.05 WT 1 month vs LXRα−/− 1 month values. #p<0.05 1 month vs 9 month values.
5.2.2 Genes involved in hepatic cholesterol metabolism

Given the crucial role LXRα plays in modulating cholesterol metabolism, we investigated genes involved in hepatic cholesterol conversion into bile acids (CYP7A1), cholesterol efflux into bile (ABCG5, ABCG8), and cholesterol biosynthesis (3-Hydroxy-3-methylglutaryl Coenzyme A Synthase [HMGCS1]).

In mice, CYP7A1 has an identified LXRE in its promoter and it is considered as a direct target gene of LXRα (118). Interestingly, a downregulation of CYP7A1 is only significant in 1 month old male LXRα⁻/⁻ mice (Figure 5.6.A). Furthermore, in LXRα⁻/⁻ mice there is an age-dependent induction (1-9 months) of CYP7A1 mRNAs in both females and males. However, in WT male mice, CYP7A1 mRNAs are significantly age-reduced from 1-9 months, whereas an opposite, but not significantly ($P = 0.05$) trend is observed in WT females.

![Figure 5.6](image)

**Figure 5.6.** Relative mRNA levels encoding genes involved in cholesterol excretion (A) and biosynthesis (B) *in vivo*. WT and LXRα⁻/⁻ male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had *ad libitum* access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol® reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. *p<0.05 WT 1 month vs LXRα⁻/⁻ 1 month values. #p<0.05 1 month vs 9 month values.
HMGCS1 is involved in cholesterol biosynthesis (26) and does not have any known LXRE in its promoter. Interestingly, the basal mRNA expression of HMGCS1 is regulated in mice studied. In 1 month old female and male LXRα−/− mice, the HMGCS1 mRNAs are significantly higher compared to WT controls (Figure 5.6.B). These levels remain higher in the LXRα−/− mice compared to WT mice in all ages (1-9 months), suggesting that LXRα may have an inhibitory effect on the basal mRNA expression of this gene. Furthermore, HMGCS1 mRNAs are significantly age-induced in WT females only.

The ABC transporters, ABCG5/G8, are involved in hepatic cholesterol efflux into the bile (26). To this date, no LXRE(s) is identified in their promoters. We demonstrate that the basal mRNA levels of ABCG5/G8 are significantly downregulated in 1 month old males compared to their WT littermates, but not in females (Figure 5.7).

![Figure 5.7. Relative mRNA levels encoding genes involved in cholesterol efflux into bile in vivo.](image)

**Figure 5.7. Relative mRNA levels encoding genes involved in cholesterol efflux into bile in vivo.** WT and LXRα−/− male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had *ad libitum* access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol® reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. *p<0.05 WT 1 month vs LXRα−/− 1 month values.
5.2.3 Genes involved in hepatic lipid metabolism

The role of LXRα in lipid metabolism is well characterized (30). Studies have shown that mice treated with LXR agonists exhibit increased hepatic lipogenesis, an effect that has largely been attributed to the LXR dependent upregulation of SREBP-1c expression (49;73;91). The SREBP-1c gene has two LXREs in its promoter (73;91), and can regulate expression of the de novo lipogenic genes FAS and SCD-1(91). However, treating SREBP-1c null mice with an LXR agonist, still results in the upregulation of lipogenic genes and an increase in FA synthesis, suggesting that additional mechanisms exist to link LXR activity and lipogenesis (119). Recently, it was demonstrated that ChREBP is a LXR target gene with two functional LXREs in its promoter, and that ChREBP also induces expression FAS and SCD-1 in agonist treated WT mice, but not in LXRα−/−β−/− mice (102). As both FAS and SCD-1 can be regulated by SREBP-1c, ChREBP and LXRα, we included SREBP-1 and ChREBP, as well as FAS and SCD-1 on the LDA card. We also included ELOVL1, ELOVL2, ELOVL5 and ELOVL6, genes involved in elongation of FAs in mammals (120) and acyl-CoA oxidase (ACO), which is involved in peroxisomal β-oxidation.

The basal mRNA expression of SREBP-1 in 1 month old mice does not seem to be dependent on LXRα, as no downregulation is demonstrated in LXRα−/− males or females (Figure 5.8). In fact, a small, but significant upregulation of SREBP-1 mRNA levels is observed in 1 month old LXRα−/− females when compared their WT littermates. Furthermore, a significant downregulation of ChREBP mRNAs is only observed in LXRα−/− males in comparison to WT males, but a similar trend is observed LXRα−/− females. A small, but significant age-induction is observed in LXRα−/− males (1-9 months).
Figure 5.8. Relative mRNA levels encoding TFs involved in lipid metabolism in vivo. WT and LXRα−/− male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had ad libitum access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol® reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. *p<0.05 WT 1 month vs LXRα−/− 1 month values. #p<0.05 1 month vs 9 month values.

The selected genes involved in de novo lipogenesis, FAS (49;93) and SCD-1 (121), have an identified LXRE in their promoter. Our results demonstrate a significant downregulation of FAS and SCD-1 mRNAs in 1 month old female and male LXRα−/− mice compared to their WT littermates (Figure 5.9). Strikingly, the basal mRNA expressions of these genes are upregulated in LXRα−/− male and female mice from 1-9 months. This age-induction is not significant for SCD-1 in LXRα−/− males (1-9 months) although a similar trend is observed.
Figure 5.9. Relative mRNA levels encoding genes involved in de novo lipogenesis in vivo. WT and LXRα−/− male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had ad libitum access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol® reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. *p<0.05 WT 1 month vs LXRα−/− 1 month values. #p<0.05 1 month vs 9 month values.

To this date, six enzymes, termed ELOVL 1-6, involved in elongation of FAs have been identified (120). They do not have any identified LXRE(s) in their promoter. We show that ELOVL6 mRNA levels are significantly downregulated in 1 month old LXRα−/− female and male mice when compared to WT controls and then significantly age-induced from 1-9 months (Figure 5.10), following the same pattern as FAS and SCD-1. The mRNA expression of ELOVL5, however is only significantly downregulated in 1 month old LXRα−/− male mice and is not age-induced from 1-9 months. Furthermore, the mRNA expression levels of ELOVL1, ELOVL2 and ACO are not regulated by LXRα or in an age-dependent fashion (results not shown).
Figure 5.10. Relative mRNA levels encoding genes involved in FA elongation in vivo. WT and LXRα\(^{-/-}\) male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had ad libitum access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol\textsuperscript{®} reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. *p<0.05 WT 1 month vs LXRα\(^{-/-}\) 1 month values. #p<0.05 1 month vs 9 month values.

5.2.4 Other metabolic genes

Several genes encoding proteins involved in general metabolism were also included on the LDA card, such as circadian genes (Brain and Muscle Aryl Hydrocarbone ARNT-like Protein [BMAL1], Clock), NRs (ER\(\alpha\), ERR\(\alpha\), GR, Hepatocyte Nuclear Factor [HNF]-4\(\alpha\), LXR\(\beta\)) and genes that are involved in different signaling cascades, such as insulin receptor substrate (Irs) 1, NFκB, sirtuin (Sirt) 1 and SREBP-1c modifiers (Insig1 and Insig2). They were included in order to look closer into potential compensatory mechanisms for the loss of the functional LXR\(\alpha\) protein in knockout animals, and also to study their mRNA expression in ageing mice (1-9 months).

Since it is known that some NRs, such as ER\(\alpha\) (122), ERR\(\alpha\) (123) PPAR\(\alpha\) (124;125), REV-ERBs (126), interact with or regulate the basal expression of circadian genes, we investigated whether LXR\(\alpha\) is involved in circadian gene regulation as well.
Strikingly, our data demonstrate that the basal mRNA expression of BMAL1 is significantly higher in 1 month old LXRα−/− mice compared to WT controls (Figure 5.11). In addition, BMAL1 mRNA expression is significantly reduced in 3 months vs 1 month old LXRα−/− mice. Furthermore, in female LXRα−/− mice, Clock mRNA levels are also significantly higher compared to WT females, although to a lesser extent than BMAL1.

Figure 5.11. Relative mRNA levels encoding genes in circadian regulation. WT and LXRα−/− male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had ad libitum access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol® reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. *p<0.05 WT 1 month vs LXRα−/− 1 month values. #p<0.05 1 month vs 3 month values.

The relative mRNA levels of investigated NRs, including LXRβ, ERRα, HNF-4α and GR are not significantly altered in mice aged 1-9 months and do not seem dependent upon LXRα (results not shown). This is also the case for genes that are involved in different signaling cascades (Irs1, NFκB, Sirt1, Insig1 and Insig2) (results not shown).
5.2.5 Sex differences

Our findings indicate that several metabolic genes are regulated differently at mRNA level by LXRα in liver in 1 month old male and female mice studied. This is demonstrated by the significant mRNA downregulation of CYP7A1 and ABCG5/G8 in LXRα−/− males only, or by the significant higher mRNA expression of GCK in LXRα−/− females only. Furthermore, ERα was selected as a positive control for sexual dimorphism. The relative mRNA levels of ERα are significantly higher expressed in female livers compared to males in both genotypes (Figure 5.12). ERα is significantly age-induced (1-9 months) at mRNA level in the liver of LXRα−/− female mice only.

**Figure 5.12. Relative mRNA levels encoding ERα.** WT and LXRα−/− male (M) and female (F) C57BL/6 mice, 1-9 months of age, were housed in a controlled environment and had *ad libitum* access to water and food (standard chow diet). N=4 in each group. Mice were euthanized, livers were excised and total RNA was isolated using TRIzol® reagent. The expression was assessed by qRT-PCR and 36B4 was used as an endogenous control to normalisation. Relative mRNA levels were related to 1 month old WT-M. Values are means ± SEM. *p<0.05 female vs male (1-9 months) values. #p<0.05 1 month vs 9 month values.

In addition to ERα, several other genes are also differently expressed in liver of females and males when 1, 3, 6 and 9 month mRNAs for specific genes are pooled together within each group. Then, the mRNA levels of some NRs (GR, LXRβ) (results not shown), as well as mRNA levels of some genes involved in glucose
metabolism (FBP-1, GLUT2) and cholesterol/lipid metabolism (ABCG5/G8, CYP7A1, HMGCS1, ELOVL5, FAS) are also significantly higher expressed in the livers of female mice compared to male mice in both genotypes. Furthermore, in WT mice only, female animals had a significant higher mRNA expression of the ELOVL6 gene, and this difference was also observed in LXRα−/− mice, however not significant.
6. Discussion

6.1 Use of animals as a model system

Whereas the ageing process in invertebrates can be markedly altered by changing the expression of a single gene (i.e. insulin-like molecules (1)), the situation in mammals is far more complex in that a multiplicity of hormonal and other factors apparently act interdependently using various signaling modalities. Non animal models have limited power to study the involvement of NRs in the ageing process at the level of the organism. To investigate the interaction between diet, genetics and ageing in humans is difficult, because a controlled diet and environment of humans question ethical issues and is expensive, as well as complicated to perform. The versatility and power of the mouse model, makes it an invaluable tool to study metabolic factors in vivo, by making it possible to examine data of interest in a physiological context and compare results between different ages and genotypes. As such, mice were selected as a model and all data presented in this thesis are based on this model. C57BL/6 mice are frequently used in metabolic research, including research of the aging process. It should be emphasized that there is a genetic variation between mouse strains, such as in lipoprotein profiles (127) and susceptibility to diet-induced atherosclerosis (128-131), with C57BL/6 as a potent model.

To further reveal the physiologic and metabolic changes during senescence and ageing in vivo, LXRα−/− and WT mice were used. A possible limitation to this model is that alternative regulatory pathways, which is not or less important in a normal situation, might compensate for the loss of a functional LXRα. LXRα−/− mice were used, as LXRα is the dominant LXR isotype expressed in liver and is the major regulator of the hepatic glucose and lipid metabolism (25;111). LXRα−/− mice have deficiencies in hepatic LXR target gene expression that are not compensated for by the small levels of LXRβ present in this tissue (81;111;132). Furthermore, both male and female mice were used in this experiment in order to unravel sexual
dimorphisms. The use of male mice in research is often preferred to avoid the hormonal fluctuations present in female mice.

The mice were fed _ad libitum_ and had free access to water throughout the experiment. This model of _ad libitum_ fed laboratory animals can be considered as a model for sedentary humans who are at risk of obesity and associated diseases. The C57BL/6 mouse strain is normally lean when fed a normal chow diet. However, as ageing continued, the male mice developed obesity (9 months).

The major part of the liver, approximately 80% by volume, is composed of hepatocytes. Other cell types include phagocytic Kupffer cells, liver adipocytes, liver sinusoidal endothelial cells, small epithelial cells, erythrocytes and leukocytes (133). Thus, it should be emphasized that the described metabolic changes occur in the whole liver tissue, not only in the hepatocytes. In addition, only a small liver piece was used to isolate total RNA and later for profiling gene expression. It is possible that gene expression vary within the different parts of the liver.

6.2 Methodology

RNA analysis
The use of TRIzol® reagent is widely used to isolate RNA from tissues and cells of human, animal and plant origin. It provides high yields and high quality RNA if done properly. Importantly, total RNA is isolated, not only the high molecular weight mRNA. However, the method has its limitations. Firstly, incomplete removal of phenols from RNA samples may cause a less efficient cDNA synthesis, as the enzyme Reverse Transcriptase is inhibited by phenols. Secondly, the presence of phenols in RNA samples may increase the risk of RNA degradation. To address this matter, the purity of all RNA samples was evaluated using the NanoDrop (described in 4.2.2).
**Protein levels and mRNA expression**

The mRNA expression profile of tissues, do not necessarily correlate to the protein levels or enzyme activity, and may therefore be insufficient by themselves for the quantitative description of biological systems (134). This evidence include discoveries of posttranscriptional mechanisms controlling the protein translation rate, the half-lives of given proteins or mRNAs (135) and the intracellular location and molecular association of the protein products of expressed genes (136). There is a growing appreciation of the role of microRNA ([miRNA] small RNAs of ~22 nucleotide bases) in post-translational regulation of protein expression. Without encoding any protein products themselves (137), they can inhibit target mRNA translation after the translation initiation step (138). In this way miRNAs can decrease translation of specific proteins, without affecting mRNA abundance. In view of these arguments, the mRNA data presented could serve as tool to further study proteome analysis, in which the protein complement expressed by a genome is investigated (139). This is a valuable analysis due to its potential to determine properties of biological systems that are not apparent by mRNA sequence analysis alone (134). Nevertheless, with the abovementioned taken into consideration, mRNA expression data are widely used as a tool to investigate gene expression.

**Probe for SREBP-1**

The probe used in qRT-PCR recognizes both subtypes of SREBP-1 (SREBP-1a and SREBP-1c). Thus, we are not able to distinguish their expression. Nevertheless, SREBP-1c is the dominant form in the adult liver and most other intact tissues, whereas SREBP-1a predominate in cultured cell lines (140), suggesting that we mainly investigated SREBP-1c expression levels. Along this line, it has been shown that the SREBP-1c:SREBP-1a ratios in mouse liver is 9:1. High ratios have also been found in human liver (141).
6.3 The role of LXR in ageing

Some studies have shown that the expression of NRs varies between sexes and throughout lifespan in rodents (142-144). LXRα and LXRβ null animals are fertile, suggesting that these two NRs are not fundamental for embryonic development. This is not the case for their heterodimer partner RXRα, whose deletion leads to early death during mouse ontogenesis caused by placental abnormalities (145). The more severe developmental phenotype of RXRα−/− mice is explained by the fact that it alters signaling of several NRs. In situ hybridization studies on mouse embryos have revealed the onset and pattern of expression of LXRs during embryogenic development. The mRNAs for both LXR isoforms are initially detected in liver 11.5 days postcoitum (d.p.c.) (144). Later in development, expression pattern of LXRs differs. At 16.5 d.p.c. LXRα is expressed in several metabolic tissues, such as in brown adipose tissue and the small intestine, whereas the expression of LXRβ is more ubiquitous, with a particular enrichment in endocrine, immunological and neuronal structures. In adult mice, the LXRs are expressed as described previously (see 1.2.1.). Furthermore, a study performed on 3 month old and 18 month old female and male Sprague-Dawley rats by Sanguino et al. (142), demonstrated that the hepatic content of LXRα protein was higher in old male rats compared to their younger littermates, but their specific binding to a LXRE oligonucleotide (5’GCTTTGGTCACTCAAGTTCAAGTTA-3’) was reduced by 60% in comparison with samples obtained from young males. The binding to LXRE was investigated by using electrophoretic mobility shift assay (EMSA). On the contrary, there was no change in hepatic LXRα protein level and binding activity in female rats.

It is known that dysregulated glucose and lipid metabolism, two metabolic pathways where LXRs play a critical role, increase the risk for several age-associated diseases (i.e. insulin resistance and T2DM, atherosclerosis, hyperlipidemia and obesity, all of which might precipitate into cardiovascular and neuropsychiatric diseases). Therefore, it is not surprising that deregulated transcription and activity of LXRs have been observed in several pathophysiological conditions which are also described
as age-related diseases, such as CVD and T2DM (146). Along this line, numerical animal studies have suggested LXRs as potential pharmaceutical targets to attenuate the development of hypercholesterolemia (i.e. atherosclerosis (77)) and T2DM (75;99). The anti-atherogenic effects may be subscribed to LXRαs regulating genes involved in the cholesterol efflux (ABCA1, ABCG1, apoE), cholesterol conversion to bile acids (CYP7A1) and cholesterol secretion into bile for excretion (ABCG5/G8) (147). Indeed, administration of potent, selective LXR agonists may reduce aortic lesion formation (148), and even lesion regression (149) in atherogenic mouse strains. The anti-diabetic effects of LXRαs have been investigated in several studies where synthetic LXR agonists decrease glucose levels (99) and improve insulin sensitivity by downregulating hepatic gluconeogenic genes, such as PEPCK and glucose 6-phosphate dehydrogenase, and promoting glucose uptake through GLUT4 in adipose tissue (75;99). Interestingly, it has been demonstrated that a functional GLUT4 protein is crucial for sustained growth, normal cellular glucose and lipid metabolism and longevity. GLUT4 knockout mice are growth retarded and exhibit a decreased longevity associated with cardiac hypertrophy and severely reduced adipose tissue deposits (150). In view of these findings, negative side effects of LXR agonist administration, such as hepatic steatosis and hypertriglyseridemia are observed in some animal studies (49;151), partly due to elevated hepatic fatty acid synthesis and VLDL secretion. The cellular benefit to increasing lipogenesis in the presence of excess free cholesterol, is not clear to this date, however, some theories have been purposed. Firstly, it may improve the ratio of cholesterol to other lipids in order to maintain plasma membrane fluidity (91). Secondly, most mammals do not ingest pure cholesterol, but rather diets containing both fat and cholesterol. The ability to store these excess calories as lipids could be beneficial (152). Either way, this enhanced lipogenesis has temporarily hampered the development of LXR agonists in the treatment of atherosclerosis and T2DM. To further investigate the impact of LXRαs in the ageing process, we studied the role of LXRα in a normal physiologic context. Untreated LXRα−/− mice and their WT littermates (females and males, aged 1-9 months), were included.
Our data demonstrate no genotype-related difference in bodyweight from 1-9 months, but in general, females are significantly smaller than males. Bodyweight in mice studied increased significantly during ageing (1-9 months). This pattern is similar to the change of organ weight (liver and WAT). Total body fat was not measured, but the reproductive fat pad may serve as an indicator for total body fat changes. Mice further aged up to 20 months, lost bodyweight after the age of 13 months (results not shown). This effect might be due to loss of appetite, hormonal changes (i.e. decline in testosterone levels with age etc.), depression, organ atrophy or other factors.

Plasma glucose levels fluctuate within each genotype group in ageing mice (1-9 months), but were significantly higher in male compared to female mice. These findings were also observed in older mice aged 9-20 months (not shown). Even though plasma glucose levels remain unchanged in mice studied, it should be mentioned that plasma glucose is considered as a promising agent for involvement in the mechanisms of ageing. This arises from the fact that glucose, in addition to being an essential tissue nutrient, may accelerate degenerative processes. Elevated plasma glucose levels have deleterious consequences, including impaired glucose tolerance, insulin resistance and increased risk of age-related diseases, such as T2DM (153). Furthermore, elevated levels of plasma glucose may cause accumulation of advanced glycation end products (AGE), which modifies the structure of macromolecules, such as proteins and DNA. These findings led Cerami et al. to propose the Glycation Hypothesis of Ageing (154). Consistent with this theory, there is now stronger evidence for the inverse relationship between AGE formation rates and species-specific lifespan. AGEs do accumulate on long-lived human proteins as a function of age (155), and to a bigger extent in diabetic individuals (156). There is also evidence that formation of AGE promotes oxidative stress and, conversely, that oxidative stress may lead to formation of AGE. Such positive feedback loops, would promote the accelerated tissue degeneration typical of ageing (157). Although numerous studies have deciphered how glucose and other nutrients can directly lead to a metabolic response, such as insulin secretion or lipogenic gene expression (158), little is known of how this is accomplished mechanistically. Glucose may be an
endogenous ligand of LXR with the same efficacy to that of oxysterols, as recently suggested by Mitro et al. (67), but this has not been proven in a physiological setting. Thus, it is tempting to question whether glucose acts through alternative pathways to modify LXR activity. Along this line, no crystal structural studies have identified a binding of glucose to LXR. Nevertheless, it is possible that glucose modifies LXR activity in vivo by other mechanisms, such as changing subcellular localization of LXRs, post-translational modification (i.e. phosphorylation) or by facilitating recruitment of coactivators that interact with LXR. In view of these theories, it is demonstrated that glucose affects subcellular translocation of LXRα in pancreatic β-cells without affecting the LXRα protein level (159). The molecular basis of how glucose affects LXR, remains to be elucidated, but could provide further evidence to how LXRs integrate hepatic glucose and lipid metabolism.

Insulin has been identified as another pro-ageing factor (2;3). In the worm Caenorhabditis elegans, the insulin signaling pathway determines the response to longevity and environmental stress (160). Results from this thesis, demonstrate that the serum insulin concentration is significantly higher in male mice of both genotypes when compared to their female littermates (1-9 months). Given that male mice of both genotypes weigh more than their female littermates, it is possible that this difference in serum insulin is caused by the difference body- and organweight (liver, WAT). Along this line, we found a positive correlation between insulin and and body- and organ weight supporting this theory. Furthermore, the serum insulin levels in male LXRα−/− mice increase significantly from 1-3 months, an effect that is also significant from 1-6 months, but not from 1-9 months. It is therefore possible that LXRα crosstalks with insulin signaling early in life, but this needs to be rigoursly tested before any conclusion can be made, especially since insulin concentrations in serum do not follow the same pattern in LXRα−/− females, where a small increase in insulin levels is only significant from 1-9 months. Nevertheless, the link between LXRs and insulin signaling is supported by the fact that LXRs, in particular LXRβ, seem to be important for pancreatic insulin secretion, and that LXR activators promote insulin secretion (161;162). This highlights the potential of LXR agonists as
insulin sensitizers, as investigated in several models (99;163-165). Furthermore, a study on C57BL/6 WT female mice given a normal diet showed that insulin levels were induced from 2-12 months (166). In contrast to this finding, insulin levels do not increase significantly in WT female mice (1-9 months) studied in this thesis. However, we demonstrate a significant induction in serum insulin levels in *ad libitum* fed WT male mice aged 1-9 months. The increase in serum insulin levels in WT males (1-9 months), combined with their unchanged plasma glucose levels may indicate an early stage of insulin resistance as more insulin is needed to keep the glucose levels within a narrow range. This is consistent with reports of increased serum insulin levels (167), impaired glucose tolerance and decreased insulin sensitivity associated with ageing (168;169), which could exacerbate the imbalance between death and survival signals and contribute to increased cell loss and apoptosis associated with ageing and senescence (170). Furthermore, long lived mouse mutants exhibit reduced circulating levels of glucose and insulin (171-173). C57BL/6J mice are believed to carry a genetic predisposition to develop T2DM. Challenging C57BL/6J mice with a high-fat diet, has previously shown to induce insulin resistance (174). Obesity can cause insulin resistance, higher circulating insulin levels, and increased incidence of diseases that might have as their basis uncontrolled insulin signaling (175;176). For example, insulin resistance is a major pathogenic factor in the development of T2DM. Along this line, there is evidence that long-lived humans have decreased insulin signaling (177). Furthermore, one of the physiological characteristics in centenarians is their greatly increased insulin sensitivity compared with younger subjects (178;179). We have previously investigated the link between insulin and LXR in regulating hepatic FA synthesis and glucose metabolism (96). Insulin induces the LXRα mRNA levels in liver, leading to an increase in steady state mRNA level of LXR target genes, such as SREBP-1c as several genes encoding enzymes in FA and cholesterol biosynthesis. In addition, LXRα−/−β−/− mice exhibit suppressed insulin-mediated induction of an entire class of lipogenic and cholesterogenic enzymes, supporting the role of LXR as insulin sensors in hepatic lipid homeostasis (96).
From this study, the levels of lipids in serum (TAG, cholesterol, FFAs) are not altered in an age-related fashion (1-9 months). There is a small, albeit not significant increase in serum TAGs of 9 months old WT males compared to 1 month old WT males. Assessing the serum lipid profile in older animals (up to 20 months), will be useful in order to investigate if serum lipids increase during ageing. Nevertheless, one can not rule out that serum lipids do not change with age, as observed in a study on aged male Brown-Norway rats (5 months vs 24 months) (167). Our results demonstrate that senescent mice present a gender-related lipid-metabolic phenotype. The levels of TAGs and cholesterol, but not FFAs are significantly lower in females compared to males in both genotypes. This sex-difference in serum lipid levels may partly be related to the higher mRNA expression of ERα (Figure 5.12) or GR (results not shown) in females, altered hepatic expression or activity of other NRs than investigated in this thesis, as well as the presence of higher estrogen levels in females (not measured). This is further discussed in “sex differences” below. Regarding genotype differences in serum lipids, we observed that LXRα−/− mice (females and males) exhibit lower levels of TAGs and cholesterol in serum compared their WT littermates, but this difference is not significant. Nevertheless, it is possible that these lipids are stored in the liver in the LXRα−/− mice, as discussed below in “LXRα regulated genes during ageing (1-9 months).”

The fact that mice were not fasted overnight before they were euthanized, one might argue that lipid values are affected by over-night dietary consumption. However, one of the aims in this study was that the mice would live as “natural” as possible with free access to food and water at all times. Fasting could interrupt the environment that the mice are used to, especially in old mice, and introduce a stress reaction with unwanted consequences. Allthough the serum values could differ if we had fasted the mice over night before sacrifice, it is likely that the trends with increased lifespan are the same in non fasted vs over night fasted mice.
*Hepatic mRNA expression of selected genes*

The tumour suppressor gene, INK4, was selected as a positive control for ageing, as the expression of this gene is known to increase throughout lifespan in mammals (117). This is consistent with the results presented (Figure 5.2). It has been proposed that this upregulation of INK4 is directly involved in a decreased self-renewal potential of some mature stem cells (180).

**LXRα regulated genes during ageing (1-9 months)**

Several metabolic genes were regulated at mRNA level by LXRα. Genes involved in glucose metabolism (GCK, GLUT2, G6P, FBP-1, PEPCK) were all regulated by the LXRα in either females or males (See 5.2.1). Along this line, the role of LXRs in regulating glucose homeostasis, has been previously evaluated (75). The dual LXRα/β agonist, GW3965, enhanced glucose tolerance in a mouse model of diet induced insulin resistance. In liver of LXR ligand treated mice, the expression of gluconeogenic enzymes, such as PEPCK and G6P, was indirectly suppressed. The alterations in PEPCK mRNAs that we demonstrated (Figure 5.2) do probably not change the gluconeogenic flux by itself, as it has been pointed out that only severe reductions (-90%) or drasic increases (+300%) of PEPCK expression are associated with changes in gluconeogenesis in mice (181). Our group has previously shown that the insulin sensitive glucose transporter, GLUT4, is directly regulated by LXR in adipose tissue (100), further underlying the role of LXR in glucose metabolism. GLUT4 is capable of transporting glucose, a substrate for de novo lipogenesis, into adipocytes. This transport is probably important in a fasting refeeding situation. Interestingly, our data indicate that the basal expression of a hepatic glucose transporter, GLUT2, is dependent upon LXRα in a normal physiological situation (Figure 5.3). This link has not previously been described.

LXRs are also important in regulating cholesterol and FA metabolism in multiple tissues, including the liver and intestine, as well as in macrophages. CYP7A1 is the rate limiting enzyme in converting excess cholesterol into bile acids. The expression of this CYP7A1 is described to be dependent on LXRα, but not LXRβ (81). This
LXRα-specific response may be due to the greater abundance of LXRα than LXRβ in the liver (85), or alternatively LXRα may have a higher activity than LXRβ in activating this specific gene. We show that the basal mRNA levels of this gene were significantly reduced in livers of LXRα−/− 1 month old males, indicating reduced cholesterol excretion (Figure 5.6.A). However, a significant upregulation of CYP7A1 is observed in LXRα−/− mice (1-9 months, both females and males), indicating that other proteins compensate for the functional loss of LXRα in regulating CYP7A1 to promote cholesterol elimination. Furthermore, there was a decrease in CYP7A1 mRNAs in 9 month old WT males. This may reflect the weakened binding of LXRα to LXRE in the liver of old male rats (18 months) compared to younger littermates (3 months), shown by others (142). They also demonstrated that the LXRα protein and its binding capacity to an LXRE, were not changed in old compared to young female rats, supporting our finding that CYP7A1 mRNA expression does not decrease in WT females. HMGCS1 is important for cholesterol biosynthesis. This gene was significantly higher expressed at mRNA level in 1 month old LXRα−/− mice of both sexes compared to their WT littermates (Figure 5.6.B). The mRNA levels also remained higher in female and male LXRα−/− mice from 3-9 months compared to WT controls. Mangelsdorf et al. has previously demonstrated that HMGCS1 mRNA levels were down regulated in response to a 2% cholesterol diet in both WT and LXRα−/− mice (81). As the regulation occurs both in mice with and devoid of LXRα, this suggests that the down regulating effect may be due to cholesterol, and not the LXRα per se. Along this line, the rate limiting enzyme in cholesterol biosynthesis, denoted HMG CoA reductase, is known to be inhibited by cholesterol (182). Our finding in LXRα−/− mice fed a standard chow diet indicates that LXRα may be important in suppressing the expression of this HMGCS1. This underlines the dysregulated cholesterol metabolism in LXRα−/− mice, as increased cholesterol synthesis (HMGCS1) seems to happen simultaneously with decreased conversion of cholesterol to bile acids (CYP7A1). Together with our data demonstrating lower serum cholesterol in LXRα−/− mice, this suggests that excess cholesterol is stored in hepatic tissue of these animals. Other genes involved in cholesterol metabolism, include the ABCG5 and ABCG8, genes important for hepatic cholesterol efflux into
bile. They are target genes of LXRs and expressed in liver and intestine (83). Strikingly, the basal mRNA expression of these genes was only lower in LXRα−/− 1 month males compared to LXRα−/− females (Figure 5.7).

FAS and SCD-1 are direct LXR target genes involved in de novo lipogenesis. Whereas FAS is involved in FA biosynthesis, SCD-1 is the rate limiting enzyme necessary for the conversion saturated FAs into monounsaturated FAs (MUFAs). Vessby et al. has previously shown that an increase in dietary MUFAs resulted in improved insulin sensitivity in healthy men and women (183), but had no effect on insulin secretion. Therefore, hepatic steatosis in which TAGs contain relatively more MUFAs, might be “healthier” than steatosis with predominantly saturated FAs containing TAGs. Moreover, we demonstrate a lower mRNA basal expression of FAS and SCD-1 in LXRα−/− mice (Figure 5.9), suggesting that LXRα is important for their expression early in life. Strikingly, a gradual increase in their mRNA levels is observed in older mice (3-9 months), illustrating that other proteins may come into play. This trend was also demonstrated by the mRNA regulation of ELOVL6 (Figure 5.10), an enzyme involved in elongation of saturated FAs or MUFAs, either ingested from the diet or synthesized in de novo lipogenesis (120). LXRβ is thought to compensate for the loss of LXRα in adipose tissue (184), but proof of this overlapping function in liver is lacking. Furthermore, the mRNA levels of LXRβ did not change in ageing mice (1-9 months, results not shown), suggesting that other proteins are responsible for the compensatory mechanism. LXR-mediated hepatic lipogenesis has been largely attributed to its ability to increase the expression and activity of the insulin-sensitive SREBP-1c gene. As both SCD-1 and FAS are under transcriptional control of both SREBP1-c (185) and LXR, one might speculate whether this is the responsible protein. However, the SREBP-1c mRNAs did not change significantly in neither genotype or between sexes in mice studied. Another potential candidate responsible for this effect, could be the glucose-sensitive transcription factor ChREBP, which is a LXR target gene (102). LXR and RXR agonists specifically induce ChREBP expression in WT mice, but not in LXRα−/−β−/− mice, a regulation dependent on two LXREs in the promoter of the ChREBP gene.
Furthermore, the ChREBP mRNA expression was almost abolished in fasted refed STZ treated LXRα–β−/− mice (Holm et al., unpublished results), indicating that the expression of ChREBP is dependent on LXRα. As such, LXRβ may activate ChREBP which then promotes the induction of lipogenic genes. Furthermore, both SREBP1-c and ChREBP are regulated to a large degree by post-translational mechanisms (102), not investigated in this study. SREBP-1c is tethered in the endoplasmatic reticulum, as well as the nuclear envelope membranes. Under low-cholesterol conditions it is released and translocated to the Golgi via SREBP cleavage-activating protein escort, followed by proteolytic cleavage by site 1 protease and site 2 protease to release the TF component of this protein (140). ChREBP is located in the cytosol by PKA-mediated phosphorylation of residues, which promotes the interaction with the protein 14-3-3 (186). High intracellular glucose levels increase the formation of xylulose 5-phosphate to increase the activity of protein phosphatase 2A to promote ChREBP nuclear localization (187).

Circadian rhythms are an important aspect of human biology. Many physiological processes display day-night rhythms, such as feeding behaviour, lipid and carbohydrate metabolism, blood pressure control and sleep-wake cycles (188). In addition circadian rhythmicity is revealed for every hormone in the circulation (189). The mammalian circadian system is organized in a hierarchy of oscillators. At the top of this hierarchy is the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. This “master” oscillator controls the circadian rhythm. However, endogenous oscillators have also been identified in peripheral organs, such as liver, heart and kidney (190). Circadian genes, such as Period1 (Per1), Per2, BMAL1, Clock and REV-ERBα are expressed not only in the SCN, but also in peripheral tissues (191). These genes are involved in regulating the circadian rhythm. BMAL1 dimerises with Clock and transactivates expression of genes involved in circadian regulation by binding to E-box elements in their promoters. In addition, it is known that the input and output pathways of the hypothalamic suprachiasmatic nuclei (SCN), as well as the function of this central pacemaker itself, change with advanced age (189). Interestingly, our data demonstrate a link between LXRα and circadian regulation,
which has not been described previously. The basal mRNA level of the circadian BMAL1 gene is significantly upregulated in both male and female 1 month old LXRα<sup>−/−</sup> mice but then significantly downregulated in 3 months compared to 1 month old LXRα<sup>−/−</sup> mice. We also observed an upregulation of Clock mRNA levels in 1 month old female LXRα<sup>−/−</sup> mice. However, this effect is less pronounced than the upregulation of BMAL1. This suggests that LXRα may be involved in suppressing the expression of circadian genes early in life (1 month). Whether this is the case for other circadian genes, would be interesting to investigate further, but is not within the scope of this thesis. In view of our finding, collaborators in the CRESCENDO project further investigated the link between LXRα and other circadian genes. Strikingly, they found that the mRNA expression levels of several other circadian genes were affected in the 1 month old LXRα<sup>−/−</sup> mice used in this thesis (preliminary results, Delaunay et al.). Nevertheless, these observations need to be further examined, but are potentially very interesting, as LXRα may influence the circadian regulation in mammals.

Taken together, these results underline the role of LXRα in glucose, cholesterol and lipid metabolism and that the regulation is differently regulated in some genes with increased life span. Strikingly, this includes regulation of genes that do not (yet) have identified LXRE(s) in their promoter, such as the case for ELOVL-6. Furthermore, the potential crosstalk with LXR and circadian genes may be the beginning of an emerging field.

**Sex differences**
When specific mRNAs for target genes of LXRα were evaluated, numerous differences between males and females were observed. Our findings indicate that several metabolic genes are regulated differently by LXRα in liver in 1 month old male and female mice studied. This is demonstrated by the significant mRNA downregulation of CYP7A1 and ABCG5/G8 in LXRα<sup>−/−</sup> males only, or by the significant higher mRNA expression of GCK in LXRα<sup>−/−</sup> females only. Furthermore, when 1, 3, 6 and 9 month mRNAs for specific genes were pooled together within
each group, females exhibited significantly higher hepatic relative mRNA levels of genes involved in glucose metabolism (FBP-1, GLUT2), as well as genes involved in cholesterol (ABCG/G8, HMGCS1, CYP7A1) and lipid metabolism, (ELOVL5/6, FAS). As both TAGs and cholesterol serum levels were lower in females, it is tempting to hypothesize that cholesterol and lipid metabolism is differently regulated under the control of LXRα between sexes, such as a higher cholesterol turnover and higher de novo lipogenesis in female animals. These findings have to be investigated further, for instance by analyzing lipids (TAGs and cholesteryl esters) in liver tissue. Along this line, the sexual dimorphism in mRNAs of genes encoding proteins involved in hepatic metabolic pathways may be subscribed to several factors. Firstly, as already mentioned, it is possible that LXRα regulates genes differently in females and males. Data supporting this theory is scarce and need to be clarified. Nevertheless, in an age-dependent manner, as shown in a study by Sanguino et al., both hepatic LXRα protein expression and activity was altered in senescent male rats, but not in female rats (142). Secondly, there might be a crosstalk with LXRs and ERs in females, but this is not well established. Thirdly, some sexual dimorphic features may be subscribed to hormonal differences. It is widely accepted that estrogen and testosterone levels differ in female and male mice, although not measured. Given the well known reduction in testosterone levels with age, it is possible that this may explain some sexual differences observed in senescent animals, as well as differences between young and old males. Fourthly, age- and sex dependent hepatic expression of other NRs may influence mRNA levels in an LXRα independent manner. From our experiments, mRNAs of ERα and GR were higher expressed in female livers.
7. Conclusion

The major activities of LXR in modulating the expression of genes involved in the glucose and lipid metabolic pathway enables the organism to adapt their fluctuating nutritional status and environmental conditions (i.e. food deprivation) by sensing the body’s energy reservoir. Since the LXRα regulate multiple aspects of glucose and lipid metabolism, it is not surprising that deregulated transcription and activity of LXRα have been observed in several pathophysiological, age-related conditions, such as CVD and T2DM. Our data indicate that LXRα is important in regulating lipid and glucose metabolism during ageing in a normal physiological setting. Several hepatic LXRα target genes seem to be regulated during the ageing process, such as the de novo lipogenic genes FAS and SCD-1. Their basal mRNA expression is dependent upon LXRα in 1 month old animals. A gradual increase in the expression of these genes, occur in LXRα/- female and male mice (1-9 months), indicating that other mechanisms/proteins come into play to compensate for the loss of LXRα. A similar trend is observed in the regulation of ELOVL6, a gene involved in elongation of FAs, which is not described as an LXRα target gene. In addition, several other genes seem to be regulated by LXRα, including CYP7A1, HMGCS1, PEPCK, and GLUT2. With the exception of GLUT2, these genes do not show any compensatory effect at mRNA level as for the genes mentioned above. Furthermore, this is the first report assessing the link between LXRα and circadian regulation. Our data show that LXRα is important in regulating the basal mRNA expression of the circadian gene, BMAL1, in 1 month old animals. As such, it is tempting to speculate that LXRα is involved in circadian regulation early in life. How this could affect ageing is potentially very interesting. Furthermore, it is known that the input and output pathways of the SCN, as well as the function of this central pacemaker itself, change with advanced age. However, the role of LXRα in circadian regulation remains to be investigated. Interestingly, this study also reveals many differences between female and male mice, regarding plasma glucose and measured serum components (i.e. insulin, TAG, cholesterol), as well as relative mRNA expression levels of specific genes (i.e.
ABCG5/G8, CYP7A1, FBP-1, GLUT2). In view of these data, it is still difficult making major concluding remarks of the role of LXRα in the ageing process, as the serum values and the gene expression pattern may change in mice older than 9 months. By studying changes in mice aged from 9 to 20 months and by including LXRβ−/− mice, as well as other tissues/organisms, a more complete picture of the link between LXRs and ageing may be provided. Although beyond the scope of this study, the suspected importance of LXRs in mammalian and especially human longevity and age-dependent diseases through control of lipid and glucose metabolism remains to be rigorously tested. In order to further evaluate this topic, the potential crosstalk with other tissues/organisms, NRs, hormones and cofactors must be addressed, as no single pathway is believed to function in an isolated manner. In fact, it is more likely that the combined forces of multiple genes embedded in many networks act together. Furthermore, it remains to be elucidated if similar differences are present in humans.

7.1 Future perspectives

- Continue ageing by including mice of 13, 16 and 20 months

- Assess lipid profile (TAGs and cholesteryl esters) in liver tissue

- Perform hyperinsulinemic euglycemic clamp in young and very old mice to describe physiological changes in insulin mediated glucose and lipid metabolism.

- Include LXRβ−/− mice to evaluate its function in ageing.

- Include LXRα−/−β−/− mice up to 3 months to obtain information of compensatory mechanisms that might come to play for loss of these receptors in the early stage of life.
- Study genes of interest in other tissues excised (heart, pancreas, WAT (epididymal and subcutaneous), musculus soleus and musculus gastrocnemius).

- In order to further validate the findings, it would be interesting to examine food consumption and other whole-animal measurements, such as total daily physiological activity, body temperature, metabolic rate and ageing pathology (i.e. glucose sensitivity, T2DM).
8. Reference List


(20) Ahren B, Pacini G. Age-related reduction in glucose elimination is accompanied by reduced glucose effectiveness and increased hepatic insulin extraction in man. J Clin Endocrinol Metab 1998 Sep;83(9):3350-6.


(68) Sparrow CP, Baffic J, Lam MH, Lund EG, Adams AD, Fu X, et al. A potent synthetic LXR agonist is more effective than cholesterol loading at inducing ABCA1


(94) Zhang Y, Yin L, Hillgartner FB. Thyroid hormone stimulates acetyl-coA carboxylase-alpha transcription in hepatocytes by modulating the composition of nuclear receptor complexes bound to a thyroid hormone response element. J Biol Chem 2001 Jan 12;276(2):974-83.


(121) Chu K, Miyazaki M, Man WC, Ntambi JM. Stearoyl-coenzyme A desaturase 1 deficiency protects against hypertriglyceridemia and increases plasma high-density lipoprotein cholesterol induced by liver X receptor activation. Mol Cell Biol 2006 Sep;26(18):6786-98.


(133) Geneser F. HISTOLOGI - på molekylærbiologisk grunnlag. 1 ed. Special Trykkeriet Viborg a-s; 2002.


Dentin R, Girard J, Postic C. Carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c): two key


