REGULATION OF XENOBIOTIC AND BILE ACID METABOLISM BY THE ANTI-AGING INTERVENTION CALORIE RESTRICTION IN MICE

By

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ABSTRACT

Calorie restriction (CR), defined as reduced calorie intake without causing malnutrition, is the best-known intervention to increase life span and slow aging-related diseases in various species. However, current knowledge on the exact mechanisms of aging and how CR exerts its anti-aging effects is still inadequate. The detoxification theory of aging proposes that the up-regulation of xenobiotic processing genes (XPGs) involved in phase-I and phase-II xenobiotic metabolism as well as transport, which renders a wide spectrum of detoxification, is a longevity mechanism. Interestingly, bile acids (BAs), the metabolites of cholesterol, have recently been connected with longevity. Thus, this dissertation aimed to determine the regulation of xenobiotic and BA metabolism by the well-known anti-aging intervention CR.

First, the mRNA expression of XPGs in liver during aging was investigated. The age-dependent mRNA profiles of 101 XPGs was determined in livers of male and female mice at 3, 6, 9, 12, 15, 18, 21, 24, and 27 months of age. Gender differences across the lifespan were observed for 52 XPGs. The mRNAs of 40 XPGs were lower in aged than young male mice, and 43 XPGs were lower in aged than young female mice. More XPGs with higher expression with age were observed in female (21 XPGs) than male (4 XPGs) mice.

To characterize BA profiles during aging, an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method recently developed by our laboratory was applied to quantify 20 individual BAs in serum and livers of male and female mice from 3 to 27 months of age. Total BAs remained constant with age in

liver, but increased 340% from 3 to 27 months in serum of female mice. In contrast, in male mice, BAs did not change in serum or liver. The higher concentrations of BAs in serum of aged female mice were likely due to female-specific increased expression of BA uptake transporters Ntcp and Oatp1b2 in liver as well as the rate-limiting enzyme for BA synthesis Cyp7a1.

Previous reports on regulation of gene expression in CR mice were inconsistent, mainly due to the large variation of diets, feeding regimes, as well as age and strain of the mice. Utilizing a "dose-response" model (0, 15, 30, or 40% CR for one month), the present dissertation investigated the regulation of XPG expression in livers by graded CR in male mice under the same study design. In general, CR (30 and 40%) altered the mRNA levels of over half of the 98 XPGs quantified (32 increased and 29 decreased). CR up-regulated some phase-I enzymes (such as Cyp4a14, Nqo1, Fmo2, and Fmo3) and numerous phase-II enzymes (many Sults, some Ugts, and most Gsts), as well as uptake transporter Oatp1a4. Furthermore, over half of the CR-induced alterations of XPG mRNA profiles appeared to be attributable to feminization of the liver.

To innovatively investigate the regulation of BA homeostasis by CR, individual BAs were quantified by UPLC-MS/MS in various compartments of the enterohepatic circulation using the "dose-response" model of CR. CR (40%) increased the BA pool size (162%). CR "dose-dependently" increased many individual BAs in serum. Notably, 40% CR increased tauro-deoxycholic acid (TDCA) over 10-fold in serum, liver, and gallbladder. The increase in BAs correlated with increased expression of BA-synthetic (Cyp7a1) and conjugating enzymes (BAL) and the ileal BA-binding protein

(Ibabp) by 40% CR. Improved glucose tolerance and lipid parameters in the CR mice correlated with altered BA composition of increased proportion of 12-hydroxylated BAs (cholic acid and DCA) but decreased muricholic acids.

In summary, this dissertation identified female-specific age-dependent changes of XPG expression and elevation of total BA concentrations in serum during aging, as well as the feminization effects of the expression of many XPGs by CR, all of which interestingly correlate with the phenomenon that females generally have longer life span than males. The current findings provide new evidence for the detoxification theory of aging and promote a better understanding of BAs as potential longevity signaling molecules, and may provide insight into drug elimination pathways that one should monitor in the elderly as well as people on diets.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
Abc	ATP-binding cassette
Adh	alcohol dehydrogenase
AhR	aryl hydrocarbon receptor
AL	*ad libitum*
Aldh	aldehyde dehydrogenase
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
Asbt	apical sodium-dependent bile acid transporter
BA	bile acid
BAL	bile acid-CoA ligase
BAT	bile acid-CoA: amino acid *N*-acyltransferase
Bcrp	breast cancer resistant protein
Bsep	bile salt export pump
CA	cholic acid
CAR	constitutive androstane receptor
CDCA	chenodeoxycholic acid
Ces	carboxylesterase
Comt	catechol-*O*-methyl transferase
DCA	deoxycholic acid
EHC	enterohepatic circulation
Ent	equilibrative nucleoside transporter
Fgf	fibroblast growth factor
Fmo	flavin-containing monooxygenase
Fox	forkhead box protein
FXR	farnesoid X receptor
Gapdh	glyceraldehyde 3-phophate dehydrogenase
GB	gallbladder
GH	growth hormone
Gst	glutathione *S*-transferase
HDCA	hyodeoxycholic acid
HNF	hepatocyte nuclear factor
Ibabp	ileal bile acid binding protein
IGF-1	insulin growth factor-1
LCA	lithocholic acid
LI	large intestine
Mate	multidrug and toxin extrusion
MCA	muricholic acid
MDCA	murideoxycholic acid

Abbreviation	Full name (Con'd)
Mdr	multidrug resistance protein
Mgst	microsomal glutathione *S*-transferase
Mrp	multidrug resistance-associated protein
mTOR	mammalian target of rapamycin
Nat	*N*-acetyltransferase
Nqo1	NAD(P)H: quinone oxidoreductase
Nrf2	nuclear factor E2-related factor 2
Ntcp	Na^+/taurocholate cotransporting polypeptide
Oat	organic anion transporter
Oatp	organic anion-transporting polypeptide
Oct	organic cation transporter
Ost	organic solute transporter
P450	cytochrome P450
Papss	3'-phosphoadenosine 5'-phosphosulfate synthetase
PGC1α	peroxisome proliferator-activated receptor γ coactivator 1-alpha
Pon	paraoxonase
Por	cytochrome P450 reductase
PPAR	peroxisome proliferator-activated receptor
PXR	pregnane X receptor
RXR	retinoid X receptor
SHP	small heterodimer partner
SI	small intestine
Sirt	Sirtuin (silent mating type information regulation 2 homolog)
Slc	solute carrier
Sult	sulfotransferase
T-BA	taurine-conjugated bile acid
U-BA	unconjugated bile acid
UDCA	ursodeoxycholic acid
Ugdh	UDP-glucose-6-dehydrogenase
Ugp	UDP-glucose pyrophosphorylase
Ugt	UDP-glucuronosyltransferase
UPLC-MS/MS	ultra-performance liquid chromatography-tandem mass spectrometry
XPG	xenobiotic processing gene

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Chapter 1: INTRODUCTION AND BACKGROUND

1.1 Introduction to aging

Aging is characterized as a progressive decline of physiological functions in various organs that ultimately leads to the functional decline or death of the whole organism. Aging is thought to be a universal, intrinsic, progressive, and deleterious process (Vina et al., 2007). The elderly, with a chronological age of 60 years and older, are expected to reach 22% of the global population by 2050, with 38% in Southern Europe and 27% in United States (United Nations Population Ageing and Development 2012). Due to this rapid increase, aging has become an important global issue.

Physiological functions lose efficiency with aging in various organs. When interacting with external insults from the environment, the elderly have a decline in capability to endure various stresses and maintain homeostasis. As listed in **Table 1.1**, the elderly have declined neural and cardiovascular functions, sarcopenia, cataracts, osteoporosis, declined vision, hearing, and fertility. The functions of liver, kidney, lung, and GI tract also are affected with aging. Moreover, the elderly have impaired glucose tolerance and more prone to develop type 2 diabetes. With an dysregulated immune system, the elderly have increased risks of auto-immune diseases and susceptibility to infections. What's more, the incidence of cancer increases exponentially with age.

Aging is a very complicated and fascinating process, which attracts numerous scientists to investigate its mechanisms and possibly interventions to slow or even reverse aging. As early as 1990, more than 300 theories of aging were raised (Medvedev, 1990). Some theories are more favored because more and more experimental evidence have been found in various species. Others are abandoned due

Table 1.1. The impact of aging on multiple organs and systems.

Targets	Impact by aging
Nervous system	neurodegeneration (decline in memory, cognition etc)
Heart	cardiac arrhythmia, arterial wall stiffening, arthrosclerosis, decrease blood flow through heart
Skeletal muscle	loss in motor co-ordination, sarcopenia
Liver	decrease in liver mass and hepatic blood flow, slow bile production and flow, impaired metabolism of some drugs
Kidney	lower glomerular filtration
Lung	decrease in pulmonary ventilation
Eyes	cataracts, loss in vision
Ears	loss in hearing
Bone	osteoporosis
Immune system	autoimmune, decrease inflammation
Reproductive system	infertility
Glucose-responsive organs	impaired glucose tolerance
Many organs	cancer
Skin	wrinkles, dermal thinning, impair wound healing
Hair	hair loss
Digestive system	decrease in stomach capacity and empty, lactose intolerance, bacterial overgrowth in small intestine

to a lack of experimental support. It is still considered unrealistic to have one theory that can explain all puzzles of aging. The main theories of aging are listed in **Table 1.2**.

The free radical theory. As one of the most prominent theories, the free radical theory is also called the oxidative stress theory, or mitochondrial theory. It is proposed that oxidative stress induced by highly reactive free radicals is responsible for oxidative damages associated with aging (Harman, 1956; Rattan, 2006; Oliveira et al., 2010). These reactive oxygen species (ROS) are generated from the electron transport chain in the mitochondria on a regular basis. Therefore, the importance of mitochondria in aging is proposed. Specifically, senescence results from damage caused by ROS to the mitochondrial genome when large amounts of ROS saturate the antioxidant capabilities of the cells (Miquel et al., 1980).

Immune senescence. The immune theory is becoming a popular theory of aging. The immune system plays critical roles in distinguishing self antigens from exogenous insults, and elimination of foreign pathogens. Immune senescence in the elderly makes them increasingly susceptible to infectious diseases such as influenza and pneumonia, as well as infection by *Clostridium* and *Staphylococcus*, which contribute to morbidity and irreversible frailty and dependency (Boraschi et al., 2010). In addition to increased susceptibility to infectious diseases, the elderly have increased risks for autoimmune diseases (Franceschi et al., 1995; Franceschi et al., 2000).

Telomere shortening. Telomeres at the end of chromosomes with repeated DNA sequences get shorter with each cell division due to problems with end replication.

Telomere shortening leads to replicative senescence and is thought to be a marker of aging (Olovnikov, 1992; De Meyer et al., 2011). Telomerase, the reverse transcriptase that specifically replicates shorter telomeres, is generally not able to counteract telomere shortening in normal somatic cells, and health life styles is associated with increased telomerase activity (De Meyer et al., 2011).

DNA damage and repair theory. Oxidative stress-induced accumulation of DNA damage can cause deleterious effects such as dysregulated gene expression and cell function, cell cycle arrest and apoptosis, as well as impaired tissue homeostasis (Smith-Sonneborn, 1979; Skinner and Turker, 2005). Although oxidative stress also damages lipids and proteins, DNA damage is especially critical for aging, due to its role as genetic material that cannot be replaced. Several DNA repair pathways have decreased efficiency with aging, such as nucleotide excision repair and non-homologous end joining repair. Moreover, impaired DNA repair pathways are associated with human progeriod syndromes (Freitas and de Magalhaes, 2011).

Antagonize pleiotropy theory. Antagonize pleiotropy indicates that genes may be selected for their beneficial effects at early stages of life, but later become deleterious with aging (Williams, 1957). It is also known as reproductive-cell cycle theory, because reproduction, as a typical example, is thought to be a costly process ensured for development, yet may be bad for longevity. Antagonism of reproduction and longevity has been supported by some evidence. For example, destroying germ-line cells can increase life span in *Drosophila* (Sgro and Partridge, 1999) and elegans (Arantes-Oliveira et al., 2002). In humans, androgen is important for the development

and function of the male reproductive system, however, prostate stimulation by testicular hormones also significantly contributes to the development of benign prostatic hyperplasia as well as aging-related prostate pathology, such as prostate cancer. In addition, correlation between castration and longevity has been identified in male pet dogs and humans (Waters et al., 2000). However, the relation of longevity and reproduction is not absolute (Weinert and Timiras, 2003). A clear-cut example of antagonistic pleiotropic gene is mammalian target of rapamycin (mTOR), the master regulator of protein synthesis, which drives development as well as aging (Blagosklonny, 2010).

Detoxification theory. A modern theory suggests that aging results from a progressive decline in detoxification of a broad spectrum of toxic metabolites generated by both xenobiotic and endobiotic metabolism (Gems and McElwee, 2005). It is coined detoxification theory in this dissertation. Longevity assurance mechanisms are proposed to primarily involve the removal of various toxic molecules that cause damage and cannot be repaired under normal conditions. The up-regulation of genes involved in phase-I and phase-II metabolism renders broad spectrum detoxification, which may be one of the best longevity assurance processes. This theory was raised upon the findings in long-lived mutants of *C. elegans (*McElwee et al., 2004), and is supported by accumulating evidences in *Drosophila* (Iqbal et al., 2009) and rodents (Amador-Noguez et al., 2007; Steinbaugh et al., 2012).

Hormesis theory. About 500 years ago, Paracelsus, the father of Toxicology, raised "the dose makes the poison". The finding of "hormesis" (Southam and Ehrlich,

1943), a biphasic dose response characterized with a low dose stimulation and a high dose inhibition, challenged the central dogma of toxicology. Low doses of drugs, toxins, and natural substances trigger multiple adaptive stress defense pathways, such as kinases, protein chaperons, phase-II enzymes, anti-oxidative and other cytoprotective proteins, which could provide protection against aging-related diseases (Calabrese et al., 2012; Calabrese, 2013).

These various theories are not completely independent of each other. For instance, mitochondrial function declines during aging, generating large amounts of free radicals and oxidative stress and thus DNA damage. DNA damage accumulation and declined DNA repair capabilities contribute to aging. Thus, the free radical theory and the DNA damage and repair theory are closely connected. Another example is the connection between detoxification theory and hormesis theory. The hormesis effects provided by some compounds are mediated by the induction of various signaling pathways, which could elicit detoxification and defense responses.

Table 1.2. Main theories of aging.

Theories	Description	References
Free radical/ Oxidative stress	Oxidative metabolism produces reactive free radicals that cause oxidative damage.	(Harman, 1956) (Rattan, 2006) (Oliveira et al., 2010)
Immune senescence	Aging-related decline of immune functions.	(Franceschi et al., 1995) (Franceschi et al., 2000) (Boraschi et al., 2010)
Telomere shortening	Telomere loss leads to cellular senescence.	(Olovnikov, 1992) (De Meyer et al., 2011)
DNA damage and repair	Oxidative damage to DNA and reduced mismatch repair lead to mutation events, and hence cancer, with aging.	(Smith-Sonneborn, 1979) (Skinner and Turker, 2005)
Antagonize pleiotropy	Genes that are beneficial during development become deleterious during aging.	(Williams, 1957) (Waters et al., 2000) (Blagosklonny, 2010)
Detoxification theory	Decline in detoxification abilities of a broad spectrum of damaging species generated by both xenobiotic and endobiotic metabolism	(Gems and McElwee, 2005) (Amador-Noguez et al., 2007) (Iqbal et al., 2009) (Steinbaugh et al., 2012)
Hormesis	Hormetic dose of xenobiotics lead to adaptation and activation of stress responsive signalings that ensure longevity.	(Calabrese et al., 2012) (Calabrese, 2013)

1.2 Models for aging research

Aging research has been promoted by various experimental models with naturally-occurring or genetically-modified mutations that lead to longevity. *Drosophila* and *C. elegans* are frequently used by gerontologists for research on the molecular mechanisms of aging, because they have short life cycle and are relatively easy for genetic modification. Recent discoveries in long-lived mutant mice have significantly promoted the area of aging research. Because it is generally thought that the most fundamental mechanisms of aging are evolutionarily conserved among species, the findings in rodents may contribute significantly to our understanding of the causes of aging and how longevity could possibly be increased in humans. Common models for aging research are explained below, with a main focus on rodents.

Dietary models. The diet known as calorie restriction (CR; also known as dietary restriction) is the most reproducible way to extend life span in mammals. As early as in Ancient Greece and Rome, the health benefits of food limitation were appreciated (Dehmelt, 2004). At the turn of the 20th century, Professor Maurice Gueniot, president of the Paris Medical Academy, is famed for living on a restricted diet and for dying at the age of 102 (Sinclair, 2005). In 1935, the first widely recognized scientific article of restricted diets and their ability to extend life span was published in rats (McCay et al., 1935). Since then, CR has been found to be effective in extending life span of various species including mammals without genetic alterations (Masoro, 2000). Similar to CR, healthy dietary patterns are effective in reducing risk for chronic diseases diets with "health" nutrition components could have beneficial effects against age-related diseases.

A classic example is the Okinawan diet, the diet of the world's longest-lived people. The traditional Okinawan diet is low in calories yet nutritionally dense with high-quality carbohydrates mostly from vegetables and fruits. Residents of Okinawa in Japan are known for their long life expectancy, high numbers of centenarians, and low risk of age-related diseases, which is thought to be related to a healthy life style, particularly the traditional low-calorie but nutritious Okinawan diet (Willcox et al., 2009). This suggests that such healthy diets may help to slow aging (Willcox et al., 2007).

Genetic models. The growth hormone/insulin-like growth factor 1 (GH/IGF-1) signaling is associated with longevity regulation. GH is secreted by the anterior pituitary gland, secreted into blood, and stimulates liver to produce IGF-1. As a primary mediator of the effects of GH, IGF-1 regulates cell growth and development. In addition to its structural similarity with insulin, IGF-1 has insulin-like effects, as it binds to the insulin receptor, at a lower affinity than IGF-1 receptor. The original finding about the connection of IGF-1 pathway and aging is in *C. elegans* with mutations in daf-2, which encodes the worm's unified insulin/IGF1 receptor (Dorman et al., 1995). Mutations that disrupt GH/IGF-1 signaling can extend life span in mice, and many have gender-divergent effect on life span **(Table 1.3)**. For example, Ames dwarf mice with a mutation in the prophet of pituitary factor-1 (Prop1) are deficient in GH and IGF-1, and have 50-70% longer mean life span (Brown-Borg et al., 1996). Snell dwarf mice with a mutation of pituitary specific transcription factor 1 (Pit1) have deficiencies in GH/IGF-1 signaling and live about 30-40% longer (Flurkey et al., 2002). In addition, Little mice, which have a mutation of GH-releasing hormone receptor, have decreased GH

production and over 20% longer life span (Flurkey et al., 2001). More evidence on the connection of GH deficiency and longevity is derived from Laron dwarf mice, which are GH receptor/GH-binding protein knockout (GHR/GHBP-KO) mice with 40-55% longer life span (Coschigano et al., 2000), and GH transgenic mice, which have increased circulating IGF-1 levels and shorter life span (Bartke et al., 2002). The critical role of IGF-1 in life span regulation is supported by long-lived IGF-1 receptor heterozygous (IGF-1R +/-) mice (Holzenberger et al., 2003) and Klotho (a hormone that represses insulin and IGF-1 receptors thus inhibiting intracellular insulin and IGF-1 signaling) transgenic mice (Kurosu et al., 2005).

Some insulin signaling mutant mice have a longer life span, such as the fat-specific insulin receptor knockout (FIRKO) mice (Bluher et al., 2003) and the insulin receptor substrate 1 (IRS-1) KO mice (Selman et al., 2008). The over-expression of antioxidant enzymes and proteins are also linked with longevity, such as the mitochondria-targeted calatase (MCAT) transgenic mice (Schriner et al., 2005) and metallothionein transgenic mice (Yang et al., 2006). Moreover, long-lived mice models with mutations in other pathways include p66shc (a 66kD isoform of Shc oncogene) KO, AC5 (adenylyl cyclase type 5) KO, αMUPA (urokinase-type plasminogen activator) transgenic, RIIβ (a regulatory subunit of protein kinase A) KO, Surf1 (a putative cytochrome c oxidase assembly factor) KO, PEPCK-C (cytosolic form of phosphoenolpyruvate carboxykinase) muscle-specific transgenic, UCP2 (uncoupling protein 2) hypocretin neuron-specific transgenic, S6K1 (ribosomal protein S6 kinase 1) KO, Agtr1a (angiotensin II receptor

type 1a) KO, and Mclk1 (mitochondrial clock abnormal protein 1) +/- mice etc (Chen et al., 2010).

Table 1.3. Some long-lived mice models with decreased GH/IGF-1 signaling.

Mice models	Genotype	Body weight	Longevity (mean life span)	References
Ames dwarf mice	Prop-1 mutant	↓ (~67%)	↑ (♂: 49%; ♀: 68%)	(Brown-Borg et al., 1996)
Snell dwarf mice	Pit-1 mutant	↓ (~67%)	↑ (♂: 26%; ♀: 42%)	(Flurkey et al., 2002)
Little mice	Ghrhr mutant	↓ (~33%)	↑ (♂: 23%; ♀: 25%)	(Flurkey et al., 2001)
Laron dwarf mice	GHR/GHBP knockout	↓ (~57%)	↑ (♂: 55%; ♀: 38%)	(Coschigano et al., 2000)
IGF-1R +/- mice	IGF-1 receptor heterozygous	↓ (<10%)	↑ (♂: 16%; ♀: 33%)	(Holzenberger et al., 2003)
Klotho mice	Klotho overexpression	N.D.	↑ (♂♀: ~20-30%)	(Kurosu et a., 2005)

Ghrhr: GH-releasing hormone receptor
GHBP: GH-binding protein
WT: wild-type
N.D.: no difference
♂: male
♀: female

1.3 Aging liver and xenobiotic metabolism

The elderly exhibit increased susceptibility to xenobiotics and often consume large amounts of drugs. Therefore, polypharmacy and high incidence of adverse drug interactions can complicate drug therapy in the elderly.

Aging is associated with multiple physiological changes, leading to pharmacokinetic changes. Liver is a major metabolic organ of the body, playing key roles in drug metabolism and detoxification. Decrease in liver mass during aging is thought to be responsible for the decline in hepatic drug metabolism, especially for low extraction drugs like phenytoin, alcohol, and theophylline, and the decrease in clearance of high extraction drugs such as hydralazine, nitrates, lidocaine, verapamil, propranolol, and morphine correlates with the decline in hepatic blood flow (Woodhouse and James, 1990). Therefore, alteration of drug metabolism likely results from physiological changes in the elderly.

1.4 Aging liver and nutrient homeostasis

Liver is the major organ of glucose and lipid metabolism. In addition, neural and hormone signals, which are presumably derived from hepatic metabolism, or metabolites themselves, markedly influences extrahepatic glucose/lipid utilization (Langhans, 2003). Overexpression of hepatic glucokinase, the key enzyme in glycolysis, can alleviate some of the metabolic disturbances triggered by insulin deficiency. This emphasizes the importance of improving hepatic glucokinase activity in diabetes (Jackerott et al., 2002). Hepatocyte nuclear factor-4 and 6 appear to play a role in activation of glucokinase (Roth et al., 2002). In addition, members of protein targeting to glycogen (PTG) family

(Gasa et al., 2000) can be used to increase glycogenic signals and lower blood glucose levels in diabetes. PPARγ coactivator 1-alpha (PGC1α) is a key modulator of hepatic gluconeogenesis (Yoon et al., 2001), and recently was shown to be involved in activation of carnitine palmitoyl-transferase-1 (CPT-1) by cAMP (Louet et al., 2002). Therefore, PGC1α is a key regulator of both glucose and lipid utilization and their coordination in liver. PGC1α is suggested as a candidate for susceptibility for type 2 diabetes (Hara et al., 2002). There is a connection of over-consumption of dietary carbohydrates and hepatic lipogenesis, involving more than 15 genes responsible for conversion of glucose to fat. A recently discovered transcription factor (Yamashita et al., 2001), called carbohydrate responsive element-binding protein (ChREBP), is activated in response to high glucose levels and it up-regulates the expression of pyruvate kinase and lipogenetic enzymes. Another fork-head box transcription factor FoxO1 is a negative regulator of insulin sensitivity in liver (Nakae et al., 2002).

Altered glucose and lipid metabolism occurs during aging. The accumulation of advanced glycation end (AGE) products generated endogenously at higher rates in diabetics due to altered glucose metabolism, may accelerate multisystem decline that occurs with aging (Semba et al., 2010). In regards to lipid metabolism, the levels of plasma high-density lipoprotein (HDL) cholesterol in elderly men are higher than those in middle-aged men (Schaefer et al., 1989; Schleich and Legros, 2004). Plasma total and low-density lipoprotein (LDL) cholesterol levels as well as triglyceride levels increase slightly after puberty and until about 50 year-old and then stabilize for the remaining years. The hepatic synthesis of very low-density lipoprotein (VLDL) and their

conversion to LDL particles is likely to increase, and the catabolism of VLDL and LDL particles is likely to decrease with age due to reduced expression of LDL receptors and activity of lipoprotein lipase (Li et al., 2008). Recently, it was reported that aging can increase the inflammatory response to excess nutrients and the vulnerability to free fatty acid-induced insulin resistance associated with aging (Einstein et al., 2010).

1.5 Calorie restriction as an anti-aging intervention

CR, defined as reduced calorie intake without causing malnutrition, is the best-known intervention that reproducibly increases both mean and maximum life span in a variety of species ranging from single cell organisms to mammals. Approximately 80 years ago, Clive Maine McCay first observed the effect of CR on life span extension in rats, promoting CR as a productive research topic in the multidisciplinary science of gerontology (McCay et al., 1935). CR increases life span in *S. cerevisiae* (Lin et al., 2000), *C. elegans* (Schulz et al., 2007), *Drosophila* (Mair et al., 2003), fish (Comfort, 1963), as well as in mammals (such as rodents and dogs) (McCay et al., 1935; Weindruch and Walford, 1982; Kealy et al., 2002). The amount by which CR extends life span in rodents depends on the extent, onset, and duration of restriction, and whether enriched diets are provided to restricted animals. Life span is progressively increased with progressive reductions in energy intake from the *ad libitum* amount up to approximately 50% reduction, beyond which further CR results in increased mortality (Mattson, 2005).

For non-human primates, a study using rhesus monkeys at the Wisconsin National Primate Research Center (WNPRC) showed that CR can blunt aging and significantly

delay the onset of age-related diseases, such as diabetes, obesity, cancer, autoimmune diseases, sarcopenia, and cardiovascular disease (Colman et al., 2008; Colman et al., 2009). In contrast, a CR study implemented in young and old age rhesus monkeys at the National Institute of Aging (NIA) showed no improvement in survival outcomes (Mattison et al., 2012). One needs to be cautious when drawing conclusions from these two studies. As described in details in **Table 1.4**, the differences in results are likely due to variations in study design, diet composition, minerals and vitamin supplementation, and genetic origins of monkeys (Ingram et al., 1990; Kemnitz et al., 1993; Ramsey et al., 2000; Kemnitz, 2011; Mattison et al., 2012).

Whether the beneficial effects of CR in lower organisms can be extrapolated to humans has been debated. Recent studies have also demonstrated beneficial effects of CR in healthy humans (as detailed in **Table 1.5**). However, much longer studies are needed to evaluate whether CR also increases the lifespan of humans. CALERIE (Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy) is a National Institute on Aging (NIA)-sponsored trial, and conducted at three clinical sites, namely Pennington Biomedical Research Center, Tufts University, and Washington University School of Medicine. The phase 2 study, which started in 2007 and ended in 2012, is the first randomized controlled trial that systematically investigated sustained CR on aging in relatively healthy, non-obese humans.

Table 1.4. Comparison between two calorie restriction (30% CR) studies in rhesus monkeys.

Differences	WNPRC			NIA								
Monkeys	Origins: Indian			Origins: both Indian and Chinese								
Sex	M	F	M	M	M	M	M	M	M	F	F	F
Sample #	30	30	16	12	12	6	8	8	14	20	20	20
Age (yrs)	8-14	8-14	6-14	0.5-1	3-5	18-25	1-4	5-9	>20	1-3	6-14	16-21
Entry year (19xx)	89	94	94	86-87	86-87	86-87	88	88	88	92	92	92
Diet composition	Purified diet (15% protein, 10% fat, 5% cellulose). Source of nutrients: protein from lactalbumin; fat from corn oil; carbohydrate from corn starch and sucrose.			NIA-1-87 diet (15% protein, 5% fat, 5% fiber). Source of nutrients: protein from wheat, corn, soybean, fish and alfalfa meal; fat from soybean oil and fish meal; carbohydrates from ground wheat, corn, and lesser sucrose. NIA diet contains components that may have an impact on health such as phytochemicals (e.g. flavonoids), ultra-trace minerals, and other unidentified elements.								
Minerals and vitamin supplement	Two different diets fed to CR and control. Only CR monkeys were supplemented with vitamin and minerals by 30%.			One diet fed to both CR and control. The diet was supplemented with additional 40% of the daily-recommended allowance of vitamin and minerals. Thus, the control monkeys were over-supplemented.								
Conclusions	CR improves survival outcomes; CR decreases the incidence of diabetes, cancer, cardiovascular disease, and brain atrophy.			CR implemented at young and older ages does not improve survival outcomes. Young-onset CR decreases the incidence of neoplasia. Old-onset CR is beneficial on metabolic parameters.								

M: male; F: female.

Table 1.5. Evidence of beneficial effects of calorie restriction in healthy humans.

Study (References)	Description	Conclusion
Okinawan diets (Kagawa, 1978)	Nutritious, low-calorie, high in vegetables and carbohydrates. ~20% fewer calories than the average of Japan.	Death rates from malignancy, brain and heart disease in Okinawa were 30-40% lower than the rest of Japan.
Fasting on alternative days (Vallejo, 1957; Stunkard, 1976)	60 healthy seniors (aged 72 yrs on average), ~35% CR for 3 yrs. Average 1500 (CR) vs 2300 (cont) kcal/day.	CR lowers hospital admissions (123 vs 219 days) and deaths (6 vs 13).
Biosphere 2 (Weyer et al., 2000; Walford et al., 2002)	8 healthy humans (7 aged 27-42 yrs and 1 aged 67 yrs; 4 men and 4 women), for 2 yrs. 1750-2100 (CR) vs 2500 (cont) kcal/day.	About 50 CR-related changes in physiologic, hematologic, hormonal, and biochemical parameters, which resemble findings in rodents and monkeys. Serum IGF-1 not changed.
Long-term CR (Fontana et al., 2004)	18 CR+18 cont (aged 25-82 yrs, 15 men and 3 women) ~20% CR for 3-15 yrs. Cont: typical American diets.	CR improves biochemical parameters in blood and decreases risk factors for cardiovascular diseases.
Long-term CR (Meyer et al., 2006)	25 CR+25 cont (aged 35-82 yrs, 21 men and 4 women) CR for 3-15 yrs. Cont: western diets.	CR decreases risk factors for cardiovascular diseases and systemic inflammation.
CALERIE-Phase 1 (Heilbronn et al., 2006; Redman et al., 2007; Redman and Ravussin, 2009)	23 healthy but overweight (BMI: 25-30 kg/m^2) humans (aged 25-50 yrs; 11 men and 12 women), ~25% CR for 6 mons	CR decreases abdominal visceral fat, but does not alter fat distribution. CR decreases body temperature, energy expenditure, serum T3, and plasma DNA damage. Serum IGF-1 not changed.
CALERIE-Phase 2 (Rickman et al., 2011; Stewart et al., 2013)	218 healthy non-obese humans (BMI: 22-28 kg/m^2, CR: cont=2:1), including 67 men (aged 21-50 yrs) and 153 women (aged 21-47 yrs), 25% CR for 2 yrs.	Highest yield possible of enrollment in the trial was achieved via the screening methods, which was more intense than disease-based clinical trials. So far, no reports are available on physiological and biochemical parameters etc.

In addition to the various beneficial effects, CR has a few adverse physical effects, such as growth retardation (for early onset of restriction), decreased reproductive functions, cold intolerance, and decreased bone density (humans) (Vitousek et al., 2004). Take reproductive function as an example, CR attenuates, delays, or abolishes fertility and fecundity in *Drosophila*, *C. elegans*, and female rats and mice (Spindler, 2010). Long-term CR suspends ovulation (Nelson et al., 1985) and affects sperm quality and counts (Brinkworth et al., 1992) in rats and mice. CR that starts after weaning delays sexual maturation, however, refeeding CR female mice at late ages turns out to rescue the reproductive senescence observed in age-matched controls evidenced by the rate of follicular depletion and oestrous cycling (Nelson et al., 1985). Limited information is available in the literature about the reproductive function by long-term CR in humans (Vitousek et al., 2004).

Table 1.6. The theories of life span extension by calorie restriction.

Theories	Evidence for	Evidence against
Develop-mental delay	CR slows development (McCay et al., 1935; McCay et al., 1975).	Adults on a CR diet later in life also have longer life span (Weindruch and Walford, 1982).
Metabolic rate	CR slows metabolic rate per unit of body mass (Krystal and Yu, 1994).	After initial drop in the first 6 weeks, CR animals have equal or higher metabolic rates than AL (Masoro, 1982).
Decrease in fat	Secretary factors by fat are important to beneficial effects on neuroendocrine system and glucose homeostasis (Barzilai and Gupta, 1999).	Little direct evidence to support that decreased fat is what extends life span in CR animals.
Reduced ROS	CR animals have less ROS-induced damages to macromolecules (Merry, 2002).	Antioxidants only slightly increase life span in flies and rodents. Longer life span in human SOD overexpressed flies is genotype- and sex-specific (Spencer et al., 2003). The SOD2-null mice do not show signs of premature aging (Van Remmen et al., 2003).
Cell survival	CR protects cells from stress-induced apoptosis (neurons, kidney, liver, and some immune cells) (Howitz and Sinclair, 2005).	CR increases rates of apoptosis or genes that promote apoptosis, especially in rapidly dividing tissues, which is thought to be a major reason why CR suppresses cancer (Zhang and Herman, 2002).
Decrease in glucose and insulin levels	High glucose levels lead to accumulation of glycation end-products associated with aging. CR decreases blood glucose and insulin (Kalant et al., 1988)	Whether lower glucose and insulin levels are the reason for the increase in life span by CR remains to be determined.
Endocrin-ology	CR decreases insulin and IGF-1 levels (Harvey et al., 2012). IGF-1 system seems to play an important role in the systemic response to CR.	The life span of dwarf mice (deficient in various endocrine factors) can be further increased by CR (Bartke et al., 2001), indicating that CR might at least partially work via alternative mechanisms.
Hormesis	CR extends the life span of yeast via hormesis (Anderson et al., 2003). Supporting evidence in worms and flies. Long-lived animals have increased stress resistance.	Hormesis is unproven in mammals.

1.6 Important mediators and signaling pathways during aging and calorie restriction

Although studies on CR started nearly 70 years ago, the underlying mechanism of how CR delays the onset of aging and extends lifespan is still unknown (Anderson et al., 2009). Theories of life span extension by CR are proposed (Sinclair, 2005), most of which are controversial **(Table 1.6)**. There are several important nutrient-sensing proteins implicated in aging and CR, including protein deacetylase sirtuins (Bordone and Guarente, 2005), nutrient sensor mTOR (Estep et al., 2009), master regulator of energy metabolism PGC-1α (Corton and Brown-Borg, 2005), insulin/IGF-1 signaling (Bergamini et al., 2003), stress defensive and survival factors FoxOs (Kim et al., 2008a), energy sensor AMPK, as well as autophagy (Rubinsztein et al., 2011) etc**.** These mediators and pathways may lead to life span extension through overlapping mechanisms.

Sirtuins. Among the mediators for CR, a family of NAD-dependent protein deacetylases termed sirtuins has received the most attention. The connection between CR and sirtuins originates from the finding that the life span extension of yeast in a low-glucose medium appears to require Sir2 (silent mating type information regulation 2) (Lin et al., 2000). The closest human ortholog to the yeast Sir2 gene is Sirt1, which deacetylates various transcription factors and coactivators, such as PGC-1 and FoxO (activation) as well as NFκB and p53 (inhibition) (Vaziri et al., 2001; Brunet et al., 2004; Motta et al., 2004; Yeung et al., 2004; Rodgers et al., 2005). Sirt1, whose expression is induced by CR, may help mediate the beneficial effects of CR in mice as well (Haigis and Guarene, 2006). The wine polyphenol resveratrol is a known Sirt1 activator, and small

molecules to activate Sirt1 are currently under clinical trials for aging-associated diseases, particularly type 2 diabetes.

mTOR. As a nutrient-responsive serine/threonine kinase, TOR integrates signals, such as nutrient and energy status, hormones, and growth factors, to regulate cell proliferation and survival, protein synthesis, autophagy etc. Inhibition of TOR signaling extends life span of yeast, *C. elegans*, *Drosophila*, and mice (McCormick et al., 2011). CR-induced low glucose and low insulin that result from CR inhibit TOR activity.

PGC1α. PGC1α, a member of PGC1 family, is a master regulator of mitochondrial biogenesis and hepatic gluconeogenesis. Similar to PGC1α, PGC1β also induces mitochondrial biogenesis and respiration. However, PGC1β has no major effects on gluconeogenesis in liver, or adaptive thermogenesis in brown adipose tissue, which is opposite to PGC1α (Handschin and Spiegelman, 2006). Sirt1 deacetylates and activates PGC1α. Under low glucose conditions in cell culture, Sirt1 induces gluconeogenic genes and hepatic glucose output through PGC1α, but does not regulate the effects of PGC1α on genes on mitochondrial biogenesis (Rodgers et al., 2005). Long-term CR increases the expression of PGC1α in various tissues (including liver) of mice (Ranhotra, 2010). CR increases PGC1α expression and mitochondrial respiration in skeletal muscle of mice (Hempenstall et al., 2011).

Insulin/IGF-1 signaling. Please see detailed discussion in previous section (**section 1.2**). Decreased insulin/IGF-1signaling is associated with an extension of lifespan in *C. elegans*, *Drosophila*, and mice (Anisimov, 2003). A crucial event of the

effects of CR in rodents and monkeys is decreased insulin and IGF-1 and it is possible that the life span extension of CR is due to decreased IGF-1 (Anisimov, 2003). However, the potential role of IGF-1 signaling in human longevity is far from clear. Neither the Biosphere nor the CALERIE studies reported a decrease in serum IGF-1 in humans after sustained CR (Walford et al., 2002; Redman and Ravussin, 2009).

FoxOs. FoxO transcription factors control the response to different types of stress, promoting cell survival via activation of genes involved in DNA repair, detoxification machinery, cell cycle progression, apoptosis inhibition, and other functions (Puig and Tjian, 2006). Key events downstream of the insulin/IGF-1 signaling include activation of IRS and inhibtion of Akt, thus inhibiting FoxOs. CR down-regulates the insulin/IGF-1 pathway and activates FOXO activity (Nakae et al., 2008). *C. elegans* mutants have increased life span depending on the FoxO transcription factor Daf-16 (Dorman et al., 1995). Activation of FoxOs in *Drosophila* and *C. elegans* can increase longevity. However, there is a lack of solid evidence for a direct role of FoxOs in mediating longevity in mammals (Greer and Brunet, 2005).

AMPK. AMPK (AMP-activated protein kinase) plays crucial roles in cellular energy homeostasis by sensitively detecting the shift in the AMP/ATP ratio. AMPK directly inhibits TOR signaling (Luo et al., 2005). Enhanced AMPK signaling is capable of extending the life span of yeast, *C. elegans*, and *Drosophila* (Tschape et al., 2002; Apfeld et al., 2004; Harkness et al., 2004). However, whether AMPK is involved in the life span extension of CR in these organisms is not clear. In rodents, fasting increases AMPK activity in skeletal muscle (de Lange et al., 2006), however, CR appears to have

little or no effect on AMPK activity in skeletal muscle or liver (Gonzalez et al., 2004; To et al., 2007).

Autophagy. Autophagy, a degradation machinery of unnecessary or dysfunctional cellular components, plays crucial roles in protein homeostasis and organelle turnover. Increasing evidence suggest connection between aging and autophagy (Rubinsztein et al., 2011), especially macroautophagy, which is induced by nutrient deprivation (Mizushima and Klionsky, 2007). Mutation of essential autophagy-related genes abolishes the life span extension in long-lived eat-2 mutants (a model of CR) of *C. elegans* (Jia and Levine, 2007) as well as *Arabidopsis* exposed to lower intensity of light (a model of CR in plants) (Minina et al., 2013). In mammals, the rate of autophagy decreases with age in various tissues and autophagy is enhanced by CR (Cuervo et al., 2005), but the role of autophagy in the life span extension of CR in mammals is not clear. Pharmacological intervention to elevate autophagy activity may provide therapeutic benefits to protein-aggregation related neurodegenerative disorders (Sarkar et al., 2009).

1.7 Calorie restriction mimetics

Increasing numbers of studies reveal that CR delays the onset of age-related diseases, improve stress resistance, and decelerate functional decline (Goodrick et al., 1982; Barger et al., 2003). Due to the difficulty to widely implement CR in humans, development of CR mimetics (CRMs) to mimic the beneficial effects of CR without reducing calorie intake has become an emerging field of research (Ingram et al., 2004).

Screening compounds that mimic CR at the transcriptional level helps to identify candidate CRMs that extend longevity. These screenings were primarily based on pathways involved in intermediary metabolism, stress response, and inflammation (Weindruch et al., 2001b). Several CRMs have been identified by this expression-based screenings, such as metformin (Dhahbi et al., 2005) and resveratrol (Howitz et al., 2003; Pearson et al., 2008). Metformin, a most widely prescribed drug to treat type 2 diabetes, is thought to work at least partially through activation of AMPK to regulate glucose uptake and metabolism (Boyle et al., 2010). Resveratrol extends the life span of yeast, *C. elegans*, and *Drosophila* in a Sir2-dependent manner, by mimicking CR (Wood et al., 2004). Although resveratrol is a CRM that can extend the life span in obese mice on a high-calorie diet (Baur et al., 2006) as well as an age-accelerated SAMP8 mice (Porquet et al., 2012), it is not effective in life span extension in wild-type mice fed a normal diet (Barger et al., 2008; Pearson et al., 2008). Furthermore, 2-deoxyglucose, which is a synthetic glucose analog that inhibits the glycolytic enzyme phosphohexose isomerase, inhibits tumor growth, decreases insulin and body temperature, and increases glucocorticoids, all of which mimic CR (Ingram et al., 2004).

However, 2-deoxyglucose has adverse chronic effects on cardiovascular function, which diminishes the enthusiasm for long-term administration (Ingram et al., 2004). In summary, CRMs are mainly screened based on the similarities of the transcriptional profiles induced by compounds and CR, and long-term studies are needed to indentify whether these candidate CRMs can extend life span.

Since 2003, NIA has started an Interventions Testing Program (ITP) to evaluate pharmacological and dietary chemicals that are potential therapeutic candidates to slow aging or prevent various aging-related diseases (Miller et al., 2007). Up to five compounds were added to the study each year and tested at three independent sites (the Jackson Laboratory, University of Michigan, and University of Texas). Whether these compounds can extend the life span and delay disease and dysfunction is tested in genetically heterogeneous mice of both genders. A summary of some compounds in testing is shown in **Table 1.7**. Rapamycin, which is an inhibitor of mTOR, extends life span in both male and female mice when given at either early or late-stage of life (Harrison et al., 2009; Miller et al., 2011). Aspirin and nordihydroguaiaretic acid (NDGA), both of which have anti-inflammatory and antioxidant activities, can extend life span in male mice, but not in female mice (Strong et al., 2008). Some polyphenolic phytochemicals, such as resveratrol, green tea extract, and curcumin, do not have statistical significant effects on life span in male or female mice (Strong et al., 2013). Interestingly, bile acids (BAs), as one of the few endogenous compounds tested, were added to the ITP in 2011, and the testing results of whether BAs can extend the life span of mice have not been published yet.

Table 1.7. Some compounds in testing for extending the longevity of mice.

Compound	Weight in the diet	Treatment start age	Test start (year)	Extend life? (Yes/No)	Publication
Aspirin	20 ppm	4 months	2004	Y (M)	(Strong et al., 2008)
NDGA	2,500 ppm	9 months	2004	Y (M)	(Strong et al., 2008)
NDGA (Phase II)	800 ppm; 2,500 ppm; 5,000 ppm	6 months (M); 6 months (M); 6 months	2010; 2010; 2010	N/A	N/A
NFP	200 ppm	4 months	2004	N (M&F)	(Strong et al., 2008)
4-OH-PBN	315 ppm	4 months	2004	N (M&F)	(Strong et al., 2008)
Rapamycin	14 ppm; 14 ppm	20 months; 9 months	2005; 2006	Y (M&F); Y (M&F)	(Harrison et al., 2009) (Miller et al., 2011)
Rapamycin (Phase II)	4.7 ppm; 14 ppm; 42 ppm	9 months; 9 months; 9 months	2009; 2009; 2009	N/A	N/A
Metformin	1,000 ppm	9 months	2011	N/A	N/A
Metformin + Rapamycin	1,000 ppm + 14 ppm	9 months	2011	N/A	N/A
Simvastatin	12 ppm; 120 ppm	10 months; 10 months	2006; 2006	N (M&F)	(Miller et al., 2011)
Resveratrol	300 ppm; 1,200 ppm; 300 ppm	12 months; 12 months; 4 months	2006; 2006; 2007	N (M&F)	(Miller et al., 2011) (Strong et al., 2013)
Oxaloacetic acid	2,200 ppm	4 months	2007	N (M&F)	(Strong et al., 2013)
Green tea extract	2,000 ppm	4 months	2007	N (M&F)	(Strong et al., 2013)
Curcumin	2,000 ppm	4 months	2007	N (M&F)	(Strong et al., 2013)
Medium Chain Triglyceride Oil	60,000 ppm	4 months	2007	N (M&F)	(Strong et al., 2013)

Table 1.7. Some compounds in testing for extending the longevity of mice (cont'd).

Compound	Weight in the diet	Treatment start age	Test start (year)	Extend life? (Yes/No)	Publication
17α-Estradiol	4.8 ppm; 14.4 ppm	10 months; 10 months	2009; 2011	N/A	N/A
Acarbose	1,000 ppm	4 months	2009	N/A	N/A
Fish Oil	15,000 ppm; 50,000 ppm	9 months; 9 months	2010; 2010	N/A	N/A
Bile Acids	5,000 ppm	5 months	2011	N/A	N/A

Each compound was given in the diet in the unit of part per million (ppm) by weight. Tests were performed in both genders, unless specially indicated.
NFP: nitroflurbiprofen; 4-PH-PBN: 4-OH-α-phenyl-*N*-*tert*-butyl nitrone; NDGA: nordihydroguaiaretic acid; M: Male; F: Female; N/A: not available (results not published).

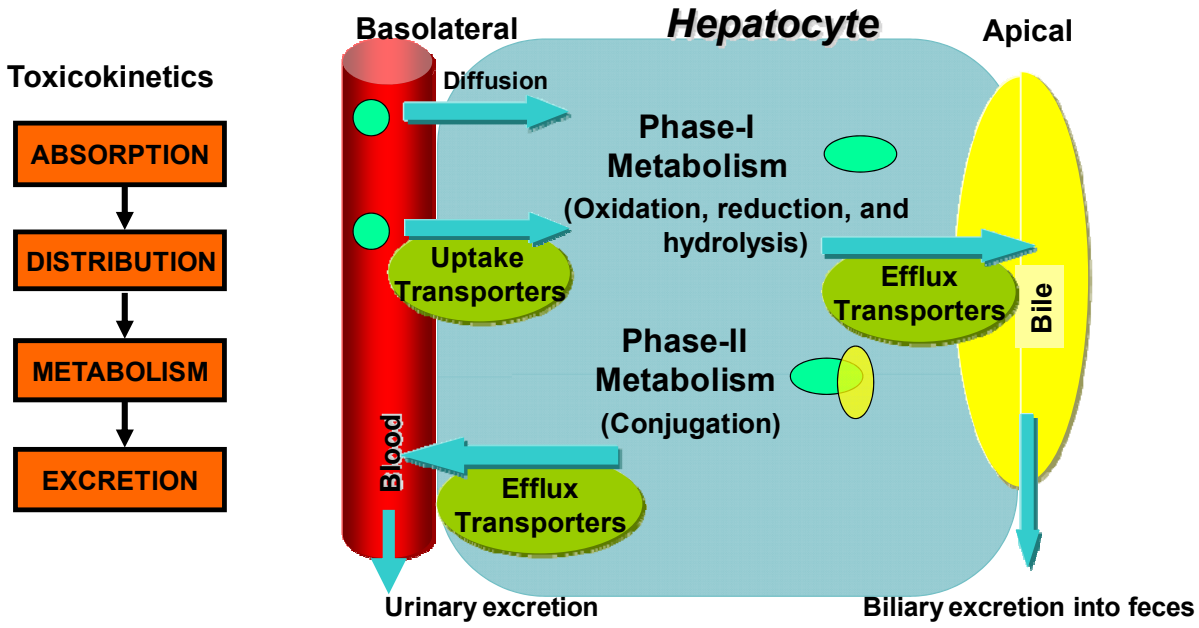
1.8 Overview of xenobiotic processing genes in liver

Liver has a high expression of xenobiotic processing genes (XPGs), including uptake transporters, phase−I enzymes, phase−II enzymes and efflux transporters. Hepatic uptake transporters play important roles in uptake of xenobiotics in liver. Uptake transporters are mainly members of the solute carrier family (Slc), including organic anion-transporting polypeptides (Oatps), organic cation transporter 1 (Oct1), organic anion transporter 2 (Oat2) and equilibrative nucleoside transporter 1 (Ent1). Phase−I enzymes catalyze oxidation, reduction, and hydrolysis reactions, including many cytochrome P450s (Cyps), flavin-containing monooxygenases (Fmos), cytochrome P450 reductase (Por), NAD(P)H:quinone oxidoreductase (Nqo), alcohol dehydrogenases (Adhs), aldehyde dehydrogenases (Aldhs), carboxylesterases (Cess) and paraoxonases (Pons) etc. Many of the phase−I metabolites can be further conjugated with sulfate, glucuronic acid, glutathione, methyl and acetyl groups catalyzed by phase−II enzymes, namely the sulfotransferases (Sults), UDP-glucuronosyltransferases (Ugts), glutathione *S*-transferases (Gsts), catechol-*O*-methyl transferase (Comt), and *N*-acetyltransferases (Nats), respectively. Phase−I reactions increase the hydrophilicity of drugs a little, whereas most phase−II reactions, except methylation and acetylation, increase their hydrophilicity a lot. After being metabolized by phase−I/II enzymes, xenobiotics become more hydrophilic and ready to be exported either into bile by canalicular efflux transporters like multidrug resistance-associate protein 2 (Mrp2), breast cancer resistant protein (Bcrp), multidrug and toxin extrusion 1 (Mate1), multidrug resistance proteins (Mdrs) 1 and 2, ATP-binding cassette transporters Abcg5 and Abcg8, or into blood to be

eliminated through urine by basolateral efflux transporters like multidrug resistant proteins (Mrps) 3, 4 and 6 and ATP-binding cassette transporter member 1 (Abca1) (Klaassen and Lu, 2008). In summary, products of XPGs play key roles in drug metabolism and detoxification in liver (a brief scheme shown in **Fig. 1.1.**).

Many of the XPGs are regulated by transcription factors (Klaassen and Aleksunes, 2010), such as the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR/NR1I3), pregnane X receptor (PXR/NR1I2), peroxisome proliferator-activated receptor alpha (PPARα/NR1C1), nuclear factor E2-related factor 2 (Nrf2), retinoid X receptor alpha (RXRα/NR2B1), and hepatocyte nuclear factors (HNF1α and HNF4α). Many of these transcription factors can be activated by xenobiotics to regulate the transcription of drug metabolizing enzymes and transporters (Klaassen and Slitt, 2005).

Fig. 1.1.



The role of liver in "xenobiotic processing". Absorption, distribution, metabolism, and excretion (or ADME), taken together is called toxicokinetics, which affects the concentrations of xenobiotics in the body and the kinetics of xenobiotic exposure to tissues, and thus their pharmacological or toxicological activities. Liver is the primary site of xenobiotic metabolism and detoxification, and the liver expresses a large number of xenobiotic processing genes. Xenobiotics in blood can enter hepatocytes either through diffusion or active uptake. Once inside hepatocytes, they can undergo phase-I metabolism, including oxidation, reduction, and hydrolysis, to become more polar metabolites. They can also undergo phase-II metabolism, when they are conjugated with sulfates, glucuronic acid, glutathione, etc. Phase-II conjugation usually increases the hydrophilicity of xenobiotics, and this favors their hepatic excretion. Hepatic efflux transporters mediate the excretion of xenobiotics and/or their metabolites into bile or back to blood.

1.9 Introduction to bile acids and the enterohepatic circulation.

Bile acids (BAs) are a major constituent of bile. BAs are amphipathic products from cholesterol metabolism in liver. Primary BAs are synthesized and conjugated in the liver are called primary BAs. Secondary BAs are formed from primary BAs by de-conjugation, de-hydroxylation, oxidation, and epimerization catalyzed by bacterial enzymes mainly in the lumen of large intestine (LI) (Ridlon et al., 2006). In mice, primary BAs include cholic acid (CA), chenodeoxycholic acid (CDCA), α-muricholic acid (αMCA), and βMCA. These BAs are converted to secondary BAs in the intestine, namely CA to deoxycholic acid (DCA), CDCA to lithocholic acid (LCA) and ursodeoxycholic acid (UDCA), αMCA and βMCA to murideoxycholic acid (MDCA), ωMCA, and hyodeoxycholic acid (HDCA) (Martin et al., 2007; Hofmann, 2009; Zhang and Klaassen, 2010).

BAs are efficiently recycled through the enterohepatic circulation (EHC) (**Fig. 1.2**). Primary BAs are synthesized and conjugated in liver, stored in the gallbladder (GB), and secreted into the intestinal lumen to facilitate the absorption of dietary fats, where the BAs are transformed into secondary BAs by intestinal bacteria. BAs are mainly reabsorbed from the ileum, and into the portal circulation. The liver extracts BAs from the portal blood, thus ending one cycle of the EHC.

BA *de novo* synthesis involves 17 different enzymes, many of which are preferentially expressed in liver (Russell, 2003). The classic synthetic pathway starts with Cyp7a1, which is the rate-limiting BA synthetic enzyme (Chiang, 1998) that catalyzes the 7α-hydroxylation of cholesterol. Cyp7a1 is the rate-limiting BA synthetic

enzyme (Chiang, 1998). The alternative pathway starts with 27-hydroxylation of cholesterol by mitochondrial Cyp27a1 (Cali and Russell, 1991), and involves 7α-hydroxylation by Cyp7b1 (Li-Hawkins et al., 2000b) to produce CDCA. Cyp8b1, a 12α-hydroxylase, is required for 12α-hydroxylation and thus CA formation (Chiang, 2003). Cyp8b1 catalyzes CA synthesis, and thus controls the ratio of CA to CDCA (Chiang, 2003). Once synthesized from cholesterol, BAs are further conjugated with taurine or glycine by bile acid-CoA ligase (BAL) and bile acid-CoA:amino acid *N*-acyltransferase (BAT) in liver. Mouse BAT is a taurine-specific conjugating enzyme (Falany et al., 1997), thus tauro-BAs are major conjugated BAs in mice.

The expression of key BA synthetic enzymes (e.g. Cyp7a1 and Cyp8b1) are well-known to be suppressed by BAs by a feedback inhibition mechanism. There are two major mechanisms of this transcriptional regulation: 1) FXR activation by BAs in liver leads to the expression of the target gene, small heterodimer partner (SHP), which suppresses the transactivation of Cyp7a1 by liver receptor homolog-1 (LRH-1) (Goodwin et al., 2000). 2) FXR activation by BAs in intestine leads to the expression of the target gene, fibroblast growth factor 15 (Fgf15), an intestinal hormone that can mediate transcriptional regulation in liver through its interaction with membrane receptor Fgfr4 (Inagaki et al., 2005). The suppression of Cyp7a1 is mediated by intestinal FXR-Fgf15 pathway, whereas the suppression of Cyp8b1 is more dependent on the FXR signaling in liver (Kim et al., 2007; Kong et al., 2012).

The EHC of BAs is promoted by BA transporters. In liver, there are BA uptake transporters, namely the Na^+/taurocholate cotransporting polypeptide (Ntcp) for

conjugated BAs (Anwer, 2004) and the organic anion transporting polypeptide 1b2 (Oatp1b2) for unconjugated BAs (Csanaky et al., 2011), and BA efflux transporter, namely the bile salt export pump (Bsep) (Wang et al., 2001). In ileum, there are BA uptake transporter, namely the apical sodium-dependent bile acid transporter (Asbt) (Dawson et al., 2003), and BA efflux transporter, namely the organic solute transporter alpha and beta (Ostα and Ostβ) (Ballatori et al., 2008; Rao et al., 2008).

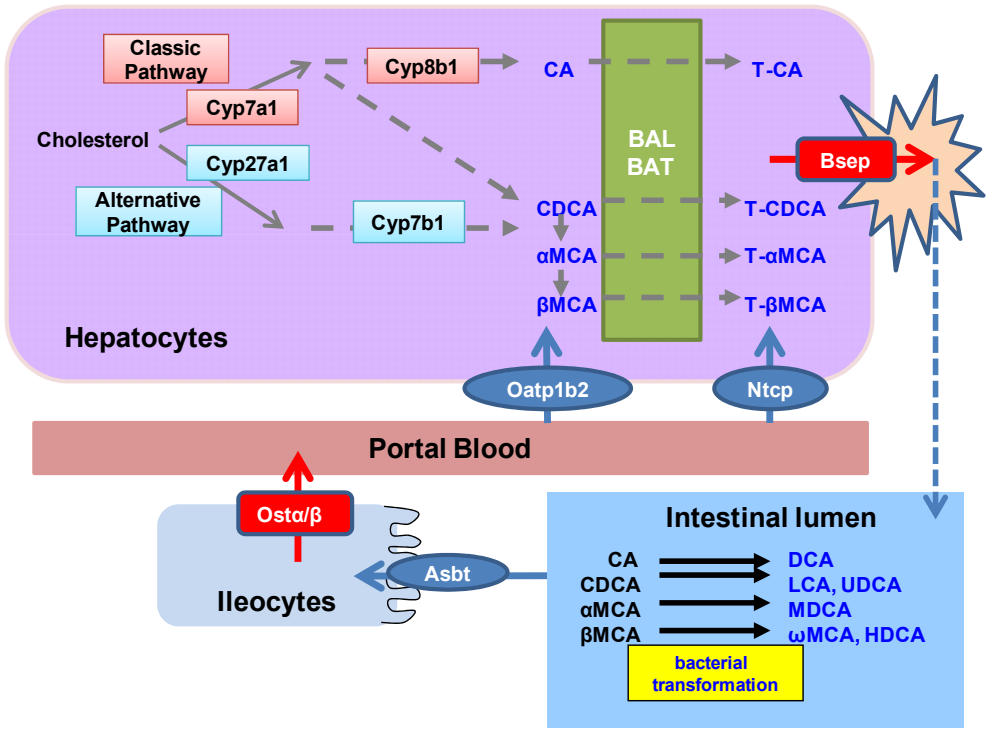
1.10 Bile acids as signaling molecules

BAs have multifaceted physiological functions. They solubilize cholesterol and other lipids in the bile to promote their elimination from the liver, and facilitate the absorption, transport, and distribution of dietary fats and lipid soluble vitamins (Hofmann and Hagey, 2008). Two BA receptors have been identified, the farnesoid X receptor (FXR/Nr1h4) (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999), which is involved in glucose and lipid metabolism (Claudel et al., 2005), and the membrane-bound receptor (TGR5/GPBAR1) (Kawamata et al., 2003), which increases energy expenditure in brown adipose tissue and muscle (Watanabe et al., 2006). In addition to the two well-known BA receptors, BAs have been shown to activate protein kinases A and C and mitogen-activated protein kinase pathways, as well as other nuclear receptors including PXR, CAR, vitamin D receptor (VDR/NR1I2), and liver X receptor (LXR/NR1H3) (Trauner et al., 2010).

BAs regulate the homeostasis of glucose (Scholmerich et al., 1985; Staels and Kuipers, 2007), lipids (Sinal et al., 2000; Sirvent et al., 2004), and energy expenditure (Watanabe et al., 2006; Li et al., 2012), in addition to their own homeostasis (Song et al.,

2011). In response to nutrient intake, glucose and insulin signaling in liver triggers alterations in BA homeostasis (Li et al., 2012). Furthermore, altered BAs and BA signaling are associated with metabolic syndromes, cardiovascular, heaptobiliary, and gastrointestinal diseases, etc (Gadaleta et al., 2010; Khurana et al., 2011; Li and Chiang, 2012). This enables BA-controlled signaling pathways to be promising novel drug targets to treat common metabolic diseases, such as obesity, type 2 diabetes, hyperlipidemia, and atherosclerosis (Houten et al., 2006).

Fig. 1.2.



Bile Acids	Abbreviation	Chemical Name
cholic acid	CA	3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid
chenodeoxycholic acid	CDCA	3α, 7α-dihydroxy-5β-cholan-24-oic acid
α-muricholic acid	αMCA	3α, 6β, 7α-trihydroxy-5β-cholan-24-oic acid
β-muricholic acid	βMCA	3α, 6β, 7β-trihydroxy-5β-cholan-24-oic acid
tauro-cholic acid	T-CA	3α, 7α, 12α-trihydroxy-5β-cholan-24-oyltaurine
deoxycholic acid	DCA	3α, 12α-dihydroxy-5β-cholan-24-oic acid
lithocholic acid	LCA	3α-hydroxy-5β-cholan-24-oic acid
ursodeoxycholic acid	UDCA	3α, 7β-dihydroxy-5β-cholan-24-oic acid
murideoxycholic acid	MDCA	3α, 6β-dihydroxy-5β-cholan-24-oic acid
ω-muricholic acid	ωMCA	3α, 6α, 7β-trihydroxy-5β-cholan-24-oic acid
hyodeoxycholic acid	HDCA	3α, 6α-dihydroxy-5β-cholan-24-oic acid

Scheme of the enterohepatic circulation of BAs in mice. Primary BAs are synthesized and conjugated in hepatocytes, and secreted into intestine to facilitate fat digestion. Cyp7a1 is the rate-limiting enzyme for BA biosynthesis. Primary BAs are conjugated mainly with taurine in mice, by conjugating enzymes BAL and BAT. BAs are excreted into the canalicular bile by Bsep. In the intestinal lumen, BAs are transformed into secondary BAs by intestinal bacteria. BAs are efficiently reabsorbed by Asbt into ileocytes and effluxed into the portal blood by Ostα/β. Ntcp and Oatp1b2 take up BAs into hepatocytes. CA, CDCA, αMCA, and βMCA are the primary BAs in mice. DCA is the secondary BA of CA, LCA and UDCA are secondary BAs of CDCA, and ωMCA, MDCA, and HDCA are secondary BAs of α,βMCA.

Chapter 2: STATEMENT OF PURPOSE

Aging is a complex physiological process characterized with progressive functional decline in various organs over time, which will eventually result in functional decline or death of the whole organism. CR is the dietary intervention that has been reproducibly shown to increase both mean and maximum life span in a variety of species ranging from unicellular organism to mammals, and effective in decreasing the incidence of aging-related diseases in humans. A modern theory suggests that aging results from declined detoxification of a broad spectrum of toxic metabolites generated by both xenobiotic and endobiotic metabolism. Up-regulation of genes involved in phase-I and phase-II metabolism, all of which are involved in detoxification, is proposed to be one of the best longevity mechanisms (Gems and McElwee, 2005). In addition, BAs, which are signaling molecules, are suggested to have anti-aging effects (Amador-Noguez et al., 2007; Gems, 2007; Goldberg et al., 2010a). Therefore, investigating the regulation of xenobiotic and BA metabolism by CR is of importance. The present dissertation is poised to fill this critical knowledge gap.

The central hypothesis of this dissertation is that CR increases the abilities of metabolism and detoxification in liver and increases BAs, both of which could contribute to longevity. The goal of this dissertation is to investigate first the effect of aging on XPG expression in liver and BA homeostasis, and secondly the regulation of XPG expression in liver and the enterohepatic circulation of BAs by CR.

Specific aim 1 was designed to characterize the effect of aging on the expression of XPGs in liver. Liver plays a major role in the metabolism and detoxification of xenobiotics (drugs and environmental chemicals) as well as endobiotics. Limited

information is available on the age-dependent expression profiles of XPGs in liver during aging. In order to reveal possible molecular mechanisms of altered xenobiotic disposition and toxicity in the elderly, multiplex suspension assays were performed to determine the age-dependent mRNA profiles of XPGs in livers of mice from 3 to 27 months of age. Totally 101 XPGs were quantified, including 7 uptake transporters, 41 phase−I enzymes, 36 phase−II enzymes, 10 efflux transporters, and 7 transcription factors. It is known in the literature that many XPGs have gender-divergent expression in liver, thus gender differences of XPG expression during aging were investigated in this aim as well.

Specific aim 2 was designed to investigate the regulation of BA homeostasis during aging. In addition to the traditional roles of BAs to facilitate cholesterol hepatic elimination of cholesterol and intestinal absorption of dietary fats and lipid-soluble vitamins, BAs have recently been recognized as signaling molecules regulating the homeostasis of glucose, lipids, and energy. Altered glucose and lipid homeostasis occurs during aging. However, information on BA levels during aging is rather limited and inconsistent. Utilizing the ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) technique, specific aim 2 determined BA profiles in serum and livers of mice from 3 to 27 months of age. The expression of genes involved in the enterohepatic circulation of BAs was quantified to mechanistically explain the age-related changes of BA concentrations and composition. Furthermore, both male and female mice were utilized to investigate whether there are any gender differences of BA homeostasis during aging.

Specific aim 3 was designed to determine the effect of CR on XPG expression in liver. The detoxification theory indicates that aging results from a progressive decline in detoxification of a broad spectrum of toxic metabolites generated by both xenobiotic and endobiotic metabolism. Longevity assurance mechanisms are proposed to primarily involve the removal of various toxic molecules that cause damage and cannot be repaired under normal conditions. Accumulating evidence in *C. elegans*, *Drosophila*, and recently in long-lived mice, such as dwarf mice, Little mice, and rapamycin-treated mice, have shown that up-regulation of genes involved in phase-I and phase-II metabolism, all of which are involved in detoxification, is associated with longevity. However, data on regulation of XPGs in CR mice are very inconsistent, mainly due to variation of diets (purified diet/non-purified, calories, and nutrition composition), different feeding regimes (percent of restriction, feeding time, and duration of CR feeding), as well as the age and strain of mice. Specific aim 3 utilized a "dose-response" model (0, 15, 30, or 40% CR for one month), to investigate the regulation of XPG expression in liver by graded CR under the same study design.

Specific aim 4 was designed to determine the effect of CR on the enterohepatic circulation of BAs. In addition to the regulatory role of BAs in nutrient and energy homeostasis, a couple of reports have recently suggested that BAs are possibly anti-aging molecules. In long-lived Little mice, elevated levels of BAs activate the expression of many XPGs possibly via BA nuclear receptor FXR, and increases resistance to xenobiotic stress, which possibly contributes to longevity. A chemical screen identified lithocholic acid (a secondary BA) as an anti-aging compound that

extended the life span of yeast. Moreover, 3-keto BA-like steroids, called dafachronic acids, have dual roles in modulating the life span of *C. elegans*. Given the promising association of BAs and longevity, studying the regulation of BAs in the best-known anti-aging intervention CR is of great importance. For the first time, BA metabolism was investigated in CR models. To systematically determine the regulation of BA enterohepatic circulation by graded CR, individual BAs were quantified by UPLC-MS/MS in serum, liver, gallbladder, as well as small and large intestine of mice fed an *ad libitum* or CR (15, 30, or 40%) diet for one month. Furthermore, the expression of genes involved in the enterohepatic circulation of BAs was quantified to mechanistically explain the CR-induced changes of BA concentrations and composition.

In summary, utilizing a "dose-response" model of CR, analytical tools, and molecular techniques, the present dissertation has investigated the alterations of XPG expression in liver and BA homeostasis with aging, and more importantly the regulation of XPG expression in liver, and the enterohepatic circulation of BAs by the best known anti-aging intervention CR. The data generated from these studies will provide further evidence to the detoxification theory of aging and further understand the possible role of BAs as longevity signaling molecules, and may provide useful information on drug elimination pathways that one should monitor in the elderly as well as people on diets.

Chapter 3: EXPERIMENTAL MATERIALS AND METHODS

3.1 Chemicals and Reagents.

The sources of individual BA standards and internal standards are described previously by Zhang and Klaassen (Zhang and Klaassen, 2010). Rabbit anti-rat Ntcp antibody (K4), which has cross-reactivity with mouse Ntcp, was a generous gift from Bruno Steiger (University Hospital, Zurich, Switzerland). A polyclonal antibody to mouse Oatp1b2 was developed in our laboratory. β-Actin antibody (ab8227) was purchased from Abcam, Inc. (Cambridge, MA). Goat anti-rabbit IgG horseradish peroxidase-linked secondary antibody was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents, unless indicated, were purchased from Sigma-Aldrich (St. Louis, MO).

3. 2 Animals and Husbandry.

All mice were housed in an AAALAC-accredited facility at the University of Kansas Medical Center, with a 14-h light/10-h dark-cycle, in a temperature-, and humidity-controlled environment and *ad libitum* access to water. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

Aging study. To investigate the effect of aging on XPG expression in liver and BA homeostasis, male (M) and female (F) C57BL/6 mice of various ages were purchased from the National Institute of Aging (Bethesda, MD) and acclimated for at least one month before tissue collections. Mice were given *ad libitum* access to standard rodent chow (Harlan Teklad 8604; Harlan Teklad, Madison, WI), until they reached 3, 6, 9, 12, 15, 18, 21, 24, and 27 months of age.

CR study. To investigate the regulation of XPG expression in liver and BA enterohepatic circulation by CR, male C57BL/6 mice (3-month old) were purchased from Charles River Laboratories (Wilmington, MA), fed with purified AIN-93M diet (TD94048, Harlan Teklad, Madison, WI) for one week, and then randomly divided into four experimental groups, namely *ad libitum* (AL), 15% CR, 30% CR, and 40% CR (n=5). All mice were housed individually, and the AL control mice were given *ad libitum* access to TD94048 diet throughout this study. The diet consumption of the AL group was recorded daily, and subsequently 85%, 70%, and 60% of this amount were given to 15%, 30%, and 40% CR groups, respectively, for one month. Feed was given between 17:00-18:00 daily. CR mice finished their daily feed within 2 hours after feeding. In order to prevent sudden weight loss, a gradual decrease in feed was provided to the mice one week before the start of CR feeding **(Table 3.1)**. In order to prevent malnutrition caused by insufficient intake of macronutrients or micronutrients, custom enriched diets TD110466, TD110467, and TD110468 were designed to feed 15%, 30%, and 40% CR groups, respectively **(Table 3.2)**. Body weights were recorded weekly.

3.3 Glucose Tolerance Test (GTT).

CR study. GTT was performed in all four groups of mice on Day 21 of CR feeding. The AL mice were fasted for 6 h starting from 8 AM, and the CR mice had finished their daily allots of feed the previous night. A single dose of D-glucose (20% solution in water; 10 mL/kg) was injected i.p. Blood was taken from tails of mice at 0, 30, 60, 90, and 120 min thereafter, and glucose concentrations were determined using a ReliOn Ultima glucose monitor (Arkray USA, Inc., Minneapolis, MN).

Table 3.1. Scheme of the CR study

	Day -7 to Day -4	Day -3 to Day 0	Day 1 to Day 30
AL	DI* (TD94048)	DI (TD94048)	DI (TD94048)
15% CR	90%DI (TD94048)	90%DI (TD94048)	85%DI (TD110466)
30% CR	85%DI (TD110466)	85%DI (TD110466)	70%DI (TD110467)
40% CR	85%DI (TD110466)	70%DI (TD110467)	60%DI (TD110468)

* DI: daily intake of food (g) of mice under AL feeding. DI was recorded daily.

Table 3.2. Custom diet formula

experiment group	AL	15% CR	30% CR	40% CR
Diet ID	TD94048	TD110466	TD110467	TD110468
Diet intake (g)	100%	85%	70%	60%
Formula (g/kg)				
Casein	140	164.7	200	233.3
L-cystine	1.8	2.1	2.6	3
Corn Starch	466	420	354	295
Maltodextrin	155	155	155	155
Sucrose	100	100	100	100
Soybean Oil	40	47.1	57.1	66.7
Cellulose	50	55	63	67.8
Mineral Mix (94049)	35	41.2	50	58.3
Vitamin Mix (94047)	10	11.8	14.3	16.7
Choline Bitartrate	2.5	2.9	3.6	4.2
TBHQ, antioxidant	0.008	0.009	0.011	0.013
Calorie (kcal/g)	3.6	3.6	3.6	3.6

All custom diets were designed based on purified AIN-93M diet.

3. 4 Sample Collection.

All sample collections were between 9:00 and 12:00 in the morning, to minimize the variations due to the circadian rhythm of XPG expression (Zhang et al., 2009) and BA metabolism (Zhang et al., 2011a).

Aging study. At 3, 6, 9, 12, 15, 18, 21, 24, and 27 months of age, mice (n=5-7) were anesthetized with pentobarbital (50 mg/kg, i.p.), and serum was collected from the suborbital vein. After cervical dislocation, liver and ileum (posterior one third of small intestine) were removed, snap-frozen in liquid nitrogen, and stored at -80°C.

CR study. Fasting plasma was collected on Day 24 of CR feeding. The AL mice were fasted for 4 h starting from 8 AM, and the CR mice had finished their daily allots of feed the previous night. All mice were anesthetized with pentobarbital (50 mg/kg), and then about 100 μL of plasma was collected by retro-orbital bleeding using heparin-coated capillary tubes. At the end of the one-month CR feeding, all mice were anesthetized with pentobarbital (50 mg/kg), and serum was collected by retro-orbital bleeding. After cervical dislocation, liver and gallbladder (GB) were removed, snap-frozen in liquid nitrogen, and stored at -80°C. The small (SI) and large intestine (LI) were flushed with 10 mL saline and the intestinal contents were collected separately on ice, and stored at -80°C. The SI and LI tissue were divided into three (duodenum, jejunum, and ileum) and two (cecum and colon) sections respectively, and collected separately, and stored at -80°C.

3.5 Total RNA Isolation.

Total RNA was isolated from liver tissue using RNA Bee reagent (Tel-Test Inc., Friendswood, TX) following the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. High quality of RNA was confirmed by visualizing two discrete rRNA bands in a RNA gel, with the 28S band doubling the intensity of the 18S band.

3.6 Multiplex Suspension Assay.

Aging study. The mRNAs of XPGs in liver were quantified in Chapter 4 by Panomics 2.0 QuantiGene Plex technology (Panomics/Affymetrix Inc., Fremont, CA), following the manufacturer's protocol. Individual gene information can be found on Panomics web site (http://www.panomics.com) with Panel numbers 21079, 21095, 21152, 21153, 21174, 21175, 21176, and 21197. **Table 3.3** listed all XPGs that were quantified by multiplex suspension assay. The mRNAs of BA-related genes were quantified in Chapter 5 by multiplex suspension assay (Panels 21095, 21151, and 21152).

CR study. The mRNAs of XPGs in liver were quantified in Chapter 6 by multiplex suspension assay (Panels 21079, 21150, 21152, 21153, 21174, 21175, and 21176). The mRNAs of genes encoding BA synthetic enzymes (Cyp7a1, Cyp8b1, Cyp27a1, and Cyp7b1) and BA transporters in liver (Ntcp, Oatp1b2, and Bsep), as well as intestinal genes (Fgf15) were also quantified in Chapter 7 by multiplex suspension assay (Panel 21150).

Fluorescence was analyzed using a Bio-Plex 200 system array reader with Luminex 100 X-MAP technology, and data were acquired using Bio-Plex data manager

software 5.0 (Bio-Rad, Hercules, CA). The mRNAs of target genes were normalized to housekeeping gene glyceraldehyde 3-phophate dehydrogenase (Gapdh).

3.7 Branched DNA Assay.

The branched DNA (bDNA) assay (Quantigene High Volume bDNA Signal Amplification Kit; Panomics/Affymetrix Inc., Fremont, CA) was used to quantify the mRNA of catechol-*O*-methyltransferase (Comt) in Chapter 4, because this gene was not included in any pre-designed panels of the multiplex suspension assay. The assay was performed as described previously (Cheng et al., 2005). Probe sets (containing capture extenders, label extenders, and blockers) specific to Comt were designed using ProbeDesigner software (Bayer Corp., Emeryville, CA), shown in **Table 3.4**.

3.8 Reverse Transcription and Real-time PCR Analysis.

Total RNA was transcribed to single-stranded cDNA using a High Capacity cDNA Reverse Transcription Kit 1001073 (Applied Biosystems, Foster City, CA), and then the cDNA products were amplified by PCR, using Power SYBR Green PCR Master Mix in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The sequences of all real-time PCR primers (Integrated DNA Technologies, Coralville, IA) are listed in **Table 3.5**.

Aging study. The mRNAs of Gapdh in livers of male and female mice at 3 and 27 months of age were quantified in Chapter 4. The mRNAs of BA conjugating enzymes (BAL and BAT) and BA transporters in ileum (Asbt, Ostα, and Ostβ) were quantified in quantified in Chapter 5, normalized to β-actin.

CR study. The mRNAs of Comt and transcription factors were quantified in Chapter 6, and normalized to β-actin. The real-time PCR primers for Comt were designed as universal primers to detect all of the three transcripts of Comt. The mRNAs of genes encoding BA conjugating enzymes (BAL and BAT), BA transporters in ileum (Asbt, Ostα, and Ostβ), ileal BA binding protein (Ibabp), and proteins involved in Cyp7a1 regulation (FXR, SHP, Fgf15, and Fgfr4) were quantified in Chapter 7, and normalized to β-actin.

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Table 3.3. Summary of XPGs quantified by multiplex suspension assay in mice liver.

Uptake Transporters	Oatp1a1, Oatp1a4, Oatp1b2, Oatp2b1, Oct1, Oat2, Ent1
Phase-I Enzymes	Cyp1a1, Cyp1a2, Cyp2b10, Cyp3a11, Cyp4a14, Por, Nqo1
	Fmo1, Fmo2, Fmo3, Fmo4, Fmo5, Fmo9, Fmo12, Fmo13
	Adh1, Adh4, Adh5, Adh6b, Adh7, Adhfe1
	Aldh1a1, Aldh1a7, Aldh1b1, Aldh2, Aldh3a2, Aldh4a1, Aldh6a1, Aldh7a1, Aldh8a1, Aldh9a1
	Ces1c, Ces1e, Ces1f, Ces1g, Ces2c, Ces3a, Aadac, Esd
	Pon1, Pon3
Phase-II Enzymes	Sult1a1, Sult1d1, Sult1e1, Sult2a1/2, Sult3a1, Sult5a1, Papss1, Papss2
	Ugt1a1, Ugt1a5, Ugt1a6a, Ugt1a9, Ugt2a3, Ugt2b1, Ugt2b34, Ugt2b35, Ugt2b36, Ugt3a1, Ugt3a2, Ugp2, Ugdh
	Gsta1, Gsta4, Gstm1, Gstm2, Gstm3, Gstm4, Gstm6, Gstp1, Gstp2, Gstt1, Gstt2, Mgst1, Mgst3
	Nat1, Nat2, Nat3
Efflux Transporters	Mrp2, Mrp3, Mrp4, Mrp6, Mate1, Mdr2, Bcrp, Abca1, Abcg5, Abcg8

(Major XPGs were quantified in this dissertation. The selection was based on our laboratory's experience and the known functions of XPGs on xenobiotic metabolism.)

Table 3.4. Oligonucleotide probes for quantifying mouse Comt mRNA by the branched DNA assay.

Comt (Accession No: NM_001111063)

Target Region	Function	Sequence
301-319	CE	ggatgcgctgctcctttgtTTTTTctcttggaaagaaagt
337-355	CE	ggtctccaggctttgcgtgTTTTTctcttggaaagaaagt
476-497	CE	caataagctcctagctccagcaTTTTTctcttggaaagaaagt
550-572	CE	tcagggttaatctccatggtgagTTTTTctcttggaaagaaagt
356-373	LE	cctccaggacgctctgggTTTTTaggcataggacccgtgtct
374-398	LE	ttctctgagcagtaggtatcaatggTTTTTaggcataggacccgtgtct
399-417	LE	cacgttcatggcccactccTTTTTaggcataggacccgtgtct
436-456	LE	ccgaatcactgcatccatgatTTTTTaggcataggacccgtgtct
457-475	LE	ccagcgagggcctgtactcTTTTTaggcataggacccgtgtct
498-515	LE	cgcacggctgagtagccaTTTTTaggcataggacccgtgtct
320-336	BL	Ttgctgcacatggcgca
418-435	BL	Ttggccttttgcgtcacc
516-531	BL	Cagcaggcgggccatt
532-549	BL	Aagcctggctccaggtgg

• CE, capture extender; LE, label extender; BL, blocker.

Table 3.5. Sequences of Real-Time PCR primers

Gene	Accession Number	Sequences
Comt *	NM_001111062,	Comt-F 5'-cacgaactcaaaccaaccaa-3'
	NM_007744, NM_001111063	Comt-R 5'-ctgttggctgctgtctcatt-3'
AhR	NM_ 013464	AhR-F 5'- accagaactgtgagggttgg-3'
		AhR-R 5'- ctcccatcgtatagggagca-3'
CAR	NM_ 009803	CAR-F 5'- ctcaaggaaagcagggtcag-3'
		CAR-R 5'- agttcctcggcccatattct-3'
PXR	AF031814	PXR-F 5'- cccatcaacgtagaggagga-3'
		PXR-R 5'-tctgaaaaaccccttgcatc-3'
PPARα	NM_ 011144	PPARα -F 5'-atgaagagggctgagcgtag-3'
		PPARα -F 5'-aaacgcaacgtagagtgctgt-3'
Nrf2	BC026943	Nrf2-F 5'-cgagatatacgcaggagaggtaaga-3'
		Nrf2-R 5'-gctcgacaatgttctccagctt-3'
BAL	NM_009512	BAL-F 5'-tgtgtgtgaaggaacctgga-3'
		BAL-R 5'-acccggacaactttgtgaag-3'
BAT	NM_007519	BAT-F 5'-tagagcacaccacgttcctg-3'
		BAT-R 5'-gcacaggctcatcaacaaga-3'
Asbt	NM_011388	Asbt-F 5'-tggaatgcagaacactcagc-3'
		Asbt-R 5'-gcaaagacgagctggaaaac-3'
Ostα	NM_145932	Ostα-F 5'-ttgtgatcaaccgcatttgt-3'
		Ostα-R 5'-ctcctcaagcctccagtgtc-3'
Ostβ	NM_178933	Ostβ-F 5'-atcctggcaaacagaaatcg-3'
		Ostβ-F 5'-ggccaagtctggtttctctg-3'
Ibabp	NM_008375	Ibabp-F 5'-caccattggcaaagaatgtg-3'
		Ibabp-R 5'-aacttgtcacccacgacctc-3'
FXR	NM_009108	FXR-F 5'-tgggtaccagggagagactg-3'
		FXR-R 5'-gtgagcgcgttgtagtggta-3'
SHP	NM_011850	SHP-F 5'-ctcatggcctctaccctcaa-3'
		SHP-R 5'-ggtcacctcagcaaaagcat-3'
Fgf15	NM_008003	Fgf15-F 5'-agaacagctccaggaccaga-3'
		Fgf15-R 5'-tccatgctgtcactctccag-3'
Fgfr4	NM_008011	Fgfr4-F 5'-ctgccagaggaagacctcac-3'
		Fgfr4-R 5'-gtagtggccacggatgactt-3'
β-actin	NM_007393	β-actin-F 5'-ggccaaccgtgaaaagatga-3'
		β-actin-R 5'-cagcctggatggctacgtaca-3'
Gapdh	NM_008084	Gapdh-F 5'-aactttggcattgtggaagg-3'
		Gapdh-R 5'-ggatgcagggatgatgttct-3'

3.9 BA Extraction.

Internal standards (40 μg/mL d_4-G-CDCA and 20 μg/mL d_4-CDCA in MeOH) were added to the samples, and BAs were extracted from serum and livers (Zhang and Klaassen, 2010), GBs (tissue with bile inside) (Zhang et al., 2011b), as well as SI and LI contents (Zhang et al., 2012b) using methods reported previously by our laboratory.

3.10 BA Quantification by Ultra-performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS).

BAs were quantified by UPLC-MS/MS using methods reported previously by our laboratory (Zhang and Klaassen, 2010). Major individual BAs quantified were TCA, TDCA, TCDCA, TLCA, TUDCA, TαMCA, TMDCA, TβMCA, TωMCA, THDCA, CA, DCA, CDCA, LCA, UDCA, αMCA, MDCA, βMCA, ωMCA, and HDCA. The concentrations of individual BAs were summed to derive the concentrations of conjugated, unconjugated, and total BAs

3.11 Plasma and Liver Lipid Parameters.

CR study. The triglycerides (TG; Pointe Scientific Inc, Canton, MI), total cholesterol (Wako Diagnostics, Richmond, VA), and nonesterified fatty acids (FAs; Wako Diagnostics, Richmond, VA) in fasting plasma collected on Day 24 of CR feeding were quantified using colorimetric assays according to the manufacturer's instructions. Liver lipids were extracted as described previously (McGrath and Elliott, 1990), and the TG, total cholesterol, and FAs in liver were quantified with the same protocol as plasma lipids.

3.12 Preparation of Crude Membrane Fractions.

Livers were homogenized in ST buffer (0.25 M sucrose, 10 mM Tris•HCl, *p*H 7.4) containing protease inhibitors and centrifuged at 100,000 g for 60 min at 4°C. The membrane pellet was rinsed and resuspended with ST buffer with protease inhibitor. Protein concentrations were determined using Pierce protein assay reagents accordingly to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL).

3.13 Western Blot Analysis.

Western blots of Ntcp and Oatp1b2 were performed as previously described with minor modifications (Csanaky et al., 2009). Primary antibodies were diluted in blocking buffer as follows: Ntcp (K4, 1:2000) and Oatp1b2 (1:1000). Membranes were stripped and reprobed with β-actin antibody (ab8227, 1:10000) as the loading control. Intensities of protein bands were determined using the Image J software (National Institutes of Health).

3.14 Statistical Analysis.

Data are presented as mean ± SEM.

Aging study. In Chapters 4 and 5, asterisks (*) represent gender differences between male and female mice, determined by Student's *t*-test ($p < 0.05$). Daggers (†) and double daggers (‡) represent age differences ($p < 0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test. In Chapter 5, pound signs (#) indicate differences of BA proportions between 3 and 27 months.

CR study. In Chapters 6 and 7, data were analyzed by one-way ANOVA, followed by Duncan's post-hoc test, differences being considered significant at $p < 0.05$

(*). In Chapter 7, to compare the BA compositions between AL and 40% CR groups, data were analyzed by Student's *t*-test, differences being considered significant at $p < 0.05$.

3.15 Hierarchical Clustering Analysis.

In Chapter 4, hierarchical clustering of XPGs that had mRNA changes with aging ($p < 0.05$, ANOVA) was performed using JMP 8.0 software (SAS Institute, Cary, NC). In Chapter 6, hierarchical clustering of XPGs that had mRNA changes by CR ($p < 0.05$, ANOVA) was also performed using JMP 8.0 software. Relatively high mRNA is represented in red color, whereas relatively low mRNA is in blue. The mRNA levels were normalized within each gene, so the relative color intensity is not comparable between genes.

Chapter 4: REGULATION OF XENOBIOTIC-PROCESSING GENE EXPRESSION IN LIVER OF MICE DURING AGING

Adapted from Fu et al., 2012. *Drug Metab Dispos*, 40, 1216-1225, with permission from the publisher.

4.1 Abstract

Aging is a physiological process characterized with progressive functional decline in various organs over time. In order to reveal possible molecular mechanisms of altered xenobiotic disposition and toxicity in the elderly, age-dependent mRNA profiles of 101 XPGs were characterized in livers of male and female mice from 3 to 27 months of age, including 7 uptake transporters, 41 phase−I enzymes, 36 phase−II enzymes, 10 efflux transporters, and 7 transcription factors. Gender differences across the lifespan (significant at five ages or more) were observed for 52 XPGs, including 15 male-predominant (e.g. Oatp1a1, Cyp3a11, Ugt1a6a, Comt, and Bcrp) and 37 female-predominant genes (e.g. Oatp1a4, Cyp2b10, Sult1a1, Ugt1a1, and Mrp3). During aging , the mRNA levels of 44% of the 101 XPGs changed in male mice and 63% changed in female mice. In male mice, mRNAs of 40 XPGs (e.g. Oatp1a1, Ces2c, Gstm4, Gstp1, and Ces1e) were lower in aged mice (over 21 months of age), whereas mRNAs of 4 XPGs (e.g. Oat2 and Gstm2) were higher in aged mice. In female mice, mRNAs of 43 XPGs (e.g. Oatp1a1, Cyp1a2, Ces1f, Sult3a1, Gstt2, Comt, Ent1, Fmo3, and Mrp6) were lower in aged mice, whereas mRNAs of 21 XPGs (e.g. Oatp1a4, Nqo1, Adh7, Sult2a1/2, Gsta1, and Mrp4) were higher in aged mice. In conclusion, 51% of the 101 XPGs have gender differences in liver mRNA levels across the lifespan of mice, and the mRNAs of 40% of the XPGs are lower in aged male mice and 43% are lower in aged female mice.

4.2 Introduction

Aging is characterized with declining physiological functions of various organs and increased incidence of multiple concomitant diseases such as diabetes, hypertension, and arthritis (Sandhiya and Adithan, 2008). The older population, described as people over the age of 65, made up 12.8% of the U.S. population during the past decade. This population is the recipient of approximately 33% of all prescription drugs and 40% of all nonprescription drugs. The elderly have been reported to have changes in the absorption, distribution, metabolism, and excretion (ADME) of many drugs, such as non-steroid anti-inflammatory drugs, antihypertensive, anticonvulsant, and psychiatric drugs (Cusack, 2004). In rodent studies, increased susceptibility to environmental chemicals has been observed during aging (Birnbaum, 1991).

Gender differences in the baseline expression of many XPGs are regulated by sex hormones or sex-dependent growth hormone patterns (Waxman and O'Connor, 2006). Gender-divergent expression regulated by growth hormone secretion was reported for Cyps (Pampori and Shapiro, 1999), Sults (Liu and Klaassen, 1996; Alnouti and Klaassen, 2006), Ugts (Buckley and Klaassen, 2009), and Gsts (Srivastava and Waxman, 1993; Knight et al., 2007), as well as xenobiotic transporters (Tanaka et al., 2005; Cheng et al., 2006; Maher et al., 2006). Because of the altered sex hormones and growth hormone levels in old age (Rudman et al., 1990; Bjornerem et al., 2004), it is important to investigate the gender-differences of XPG expression during aging.

Genome-wide studies of gene expression profiling have accelerated the research on transcriptional alterations by CR. Most of the alterations in skeletal muscle of mice

during aging were completely or partially prevented by CR by a metabolic shift toward increased protein turnover and decreased macromolecular damage (Lee et al., 1999). The same group showed CR selectively attenuated age-associated induction of genes involved in inflammatory and stress responses in the brain (Lee et al., 2000). CR was also shown to result in a 19% global inhibition of age-related changes in gene expression in mouse hearts (Lee et al., 2002).

Previous studies concerning age-dependent expression of xenobiotic metabolism genes have been restricted to only a couple of age groups (Handler and Brian, 1997; Wauthier et al., 2004; Mori et al., 2007; Lee et al., 2008), and thus possible changes of XPG expression could have been missed. Previous reports were primarily in rats, with limited data available for mice. Mice are a common laboratory model due to the availability of well-characterized mouse genome and genetically engineered strains. In addition, previous studies seldom report gender differences of XPG mRNA levels during aging. Taken together, the present study was designed to investigate the comprehensive age-dependent mRNA profiles of XPGs in livers of both male and female C57BL/6 mice with aging.

4.3 Results

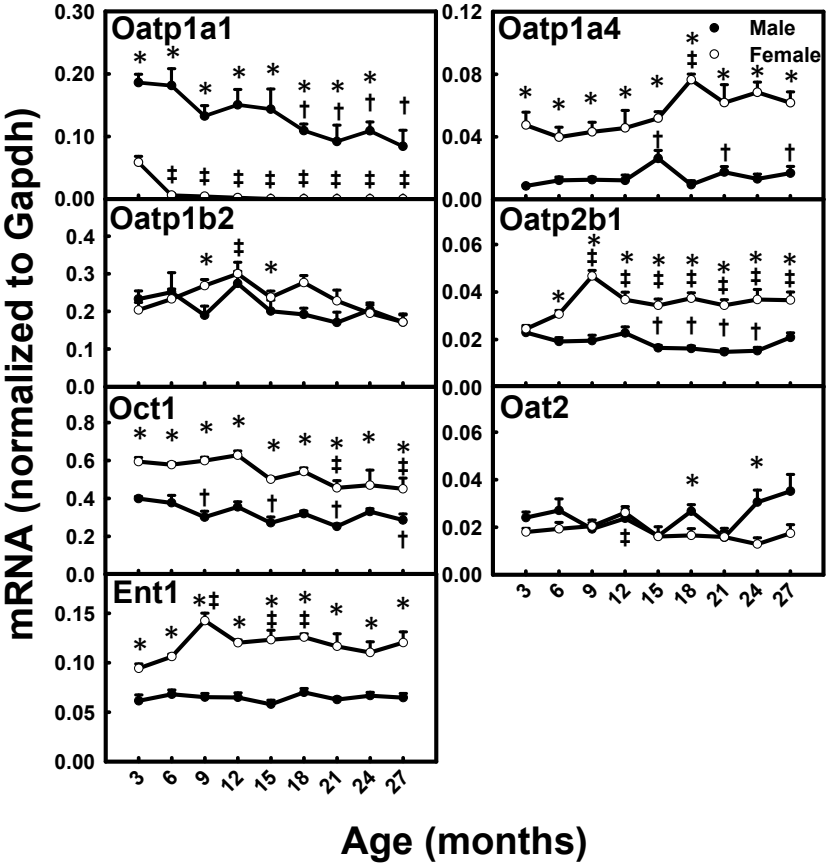
In the present study, data at 3 and 27 months of age were compared most often, because they represent young adult and senescent ages respectively. The mRNA levels of XPGs were normalized to Gapdh, which is the most commonly used house-keeping gene. The mRNA of Gapdh was quantified by real-time PCR. Gapdh mRNA remained relatively constant during aging and did not have gender differences (data not shown) (Fu et al., 2010b).

4.3.1 Uptake Transporters during Aging.

Among the uptake transporters that had decreased mRNAs with age, Oatp1a1 mRNA decreased most markedly between 3 and 27 months of age (M: 55%; F: 100%), and Oct1 mRNA decreased slightly between 3 and 27 months (M: 28%; F: 24%) **(Fig. 4.1)**. In contrast, Oatp1a4 mRNA increased 96% between 3 and 27 months in male mice, and 30% between 3 and 18 months in female mice. Oatp2b1 mRNA at 9-27 months was higher than 3 months in female mice, whereas in male mice the changes were not obvious. Ent1 mRNA increased from 3 to 9 months and tended to decrease thereafter in female mice, whereas in male mice it remained constant. The mRNA levels of Oatp1b2 and Oat2 remained relatively constant with aging. The mRNA levels of most uptake transporters had significant gender differences across the lifespan, including male-predominant Oatp1a1 and female-predominant Oatp1a4, Oatp2b1, Oct1, and Ent1. Oatp1a1 mRNA was more than 300% higher in male than female mice. In contrast, Oatp1a4 mRNA was more than 300% higher in female than male mice. The

mRNAs of Oatp2b1, Oct1, and Ent1 were all twice as high in female as those in male mice.

Fig. 4.1.



The mRNA profiles of uptake transporters with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test (p < 0.05). Daggers (†) and double daggers (‡) represent age differences (p < 0.05) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

4.3.2 Phase−I Xenobiotic Metabolizing Enzymes during Aging.

With aging, the mRNAs of Cyp1a1 (M and F) and Cyp1a2 (M and F) decreased approximately 50% between 3 and 27 months of age **(Fig. 4.2)**. Cyp3a11 mRNA decreased about 50% between 3 and 27 months in female mice, whereas it remained relatively constant with age in male mice. Interestingly, Nqo1 mRNA level remained relatively constant with age in male mice, but doubled between 3 and 27 months in female mice. The mRNA levels of Cyp2b10, Cyp4a14, and Por remained relatively constant with aging in both genders. Most Cyps had female-predominant mRNA patterns across the lifespan: the mRNAs of Cyp2b10 (10-fold) and Cyp4a14 (8-fold) were higher in female than male mice. The mRNAs of Cyp1a1, Por, and Nqo1 were also higher in female mice. Cyp3a11 was the only Cyp gene with a male-predominant mRNA pattern.

The mRNA levels of Fmos did not change much with aging **(Fig. 4.3)**. The mRNAs of Fmo2, 3, and 4 had a moderate increase between 3 and 27 months in female mice. Fmo4 mRNA in male mice also increased moderately between 3 and 27 months of age. The mRNA levels of a number of Fmos had a female-predominant pattern across the lifespan. Remarkably, Fmo3 mRNA was about 200-fold higher in female than male mice. In addition, higher mRNAs in females were also observed for Fmo1, Fmo2, and Fmo4. In contrast, Fmo5 mRNA level was male-predominant across the life span.

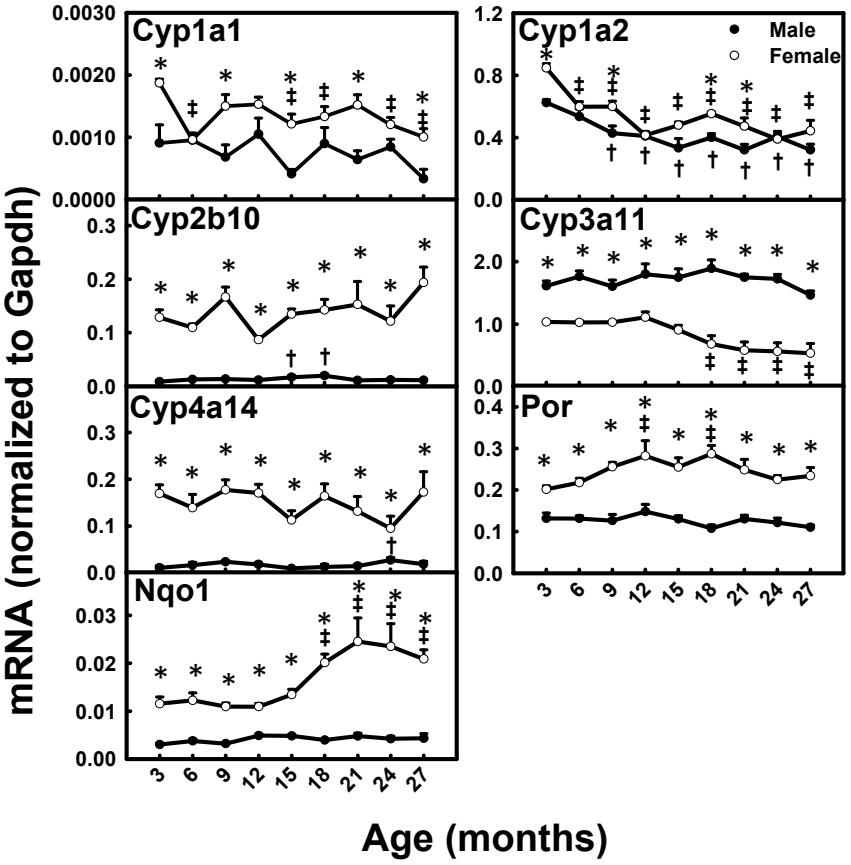
Adh4 mRNA decreased about 50% between 3 and 27 months of age in female mice **(Fig. 4.4)**. Interestingly, Adh6b decreased 29% in male mice and increased 16% in female mice between 3 and 27 months. The mRNA of Adh7 increased 93% in female

mice between 3 and 18 months. The mRNA levels of Adh1, Adh5, and Adhfe1 did not change much with aging. Most Adh mRNA levels did not show a consistent gender-divergent pattern across the lifespan. Adh4 mRNA level was male-predominant, whereas Adh6b was female-predominant after one year old.

Aldh1a1 mRNA at 6-27 months was 33% lower than 3 months in female mice **(Fig. 4.5)**. The mRNA of Aldh1a7 increased between 3 and 15 months of age in male mice. The mRNAs of Aldh2 and Aldh9a1 increased between 3 and 12 months and decreased thereafter in both genders. The mRNA levels of the remaining Aldhs remained relatively constant with aging. A number of the Aldh mRNA levels had a female-predominant pattern across the lifespan, including Aldh3a2, 4a1, and 6a1, which were 100% higher in female than male mice. Interestingly, Aldh7a1 mRNA was higher in male than female mice only between 15 and 27 months.

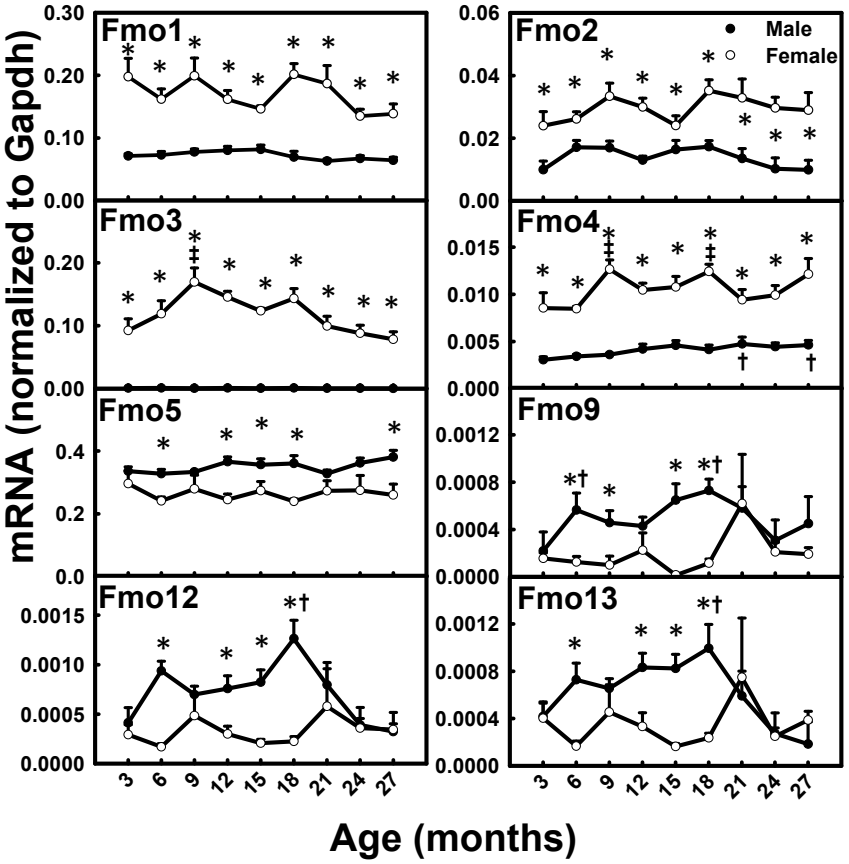
For many Cess, such as Ces1c, Ces1e, and Esd, the highest mRNAs were observed when the mice were about 12 months of age and decreased thereafter **(Fig. 4.6)**. Decreased mRNAs were observed for Ces1f (F: 49%), Ces2c (M: 60%; F: 49%), and Ces3a (F: 75%) between 3 and 27 months of age. Most of the Ces and Pon mRNA levels did not show gender-divergent patterns, except Ces2c and Ces3a, which had a male-predominant mRNA pattern, which was 200% higher in male than female mice.

Fig. 4.2.



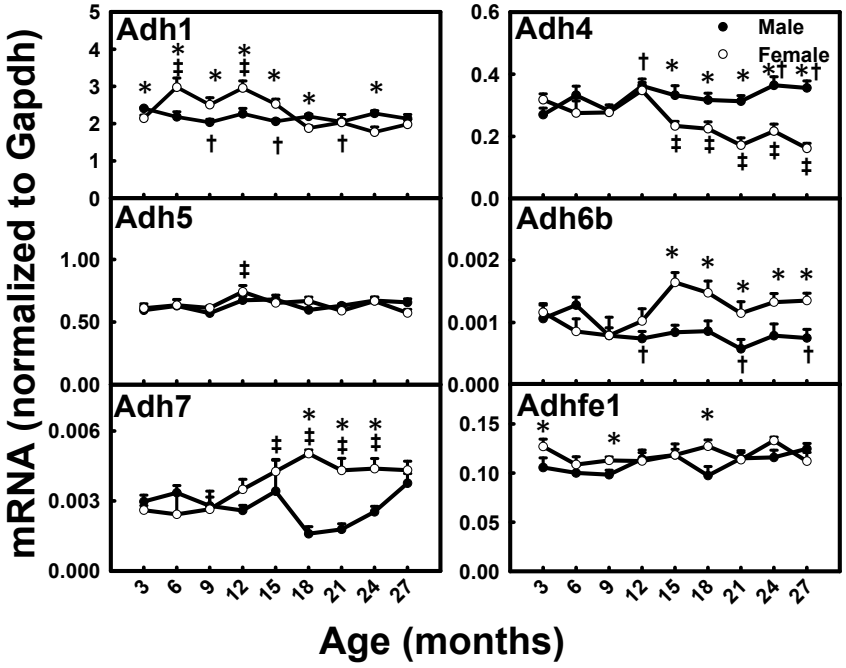
The mRNA profiles of major cytochrome P450s (P450s), P450 reductase (Por), and NAD(P)H:quinone oxidoreductase (Nqo1) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test (p < 0.05). Daggers (†) and double daggers (‡) represent age differences (p < 0.05) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

Fig. 4.3.



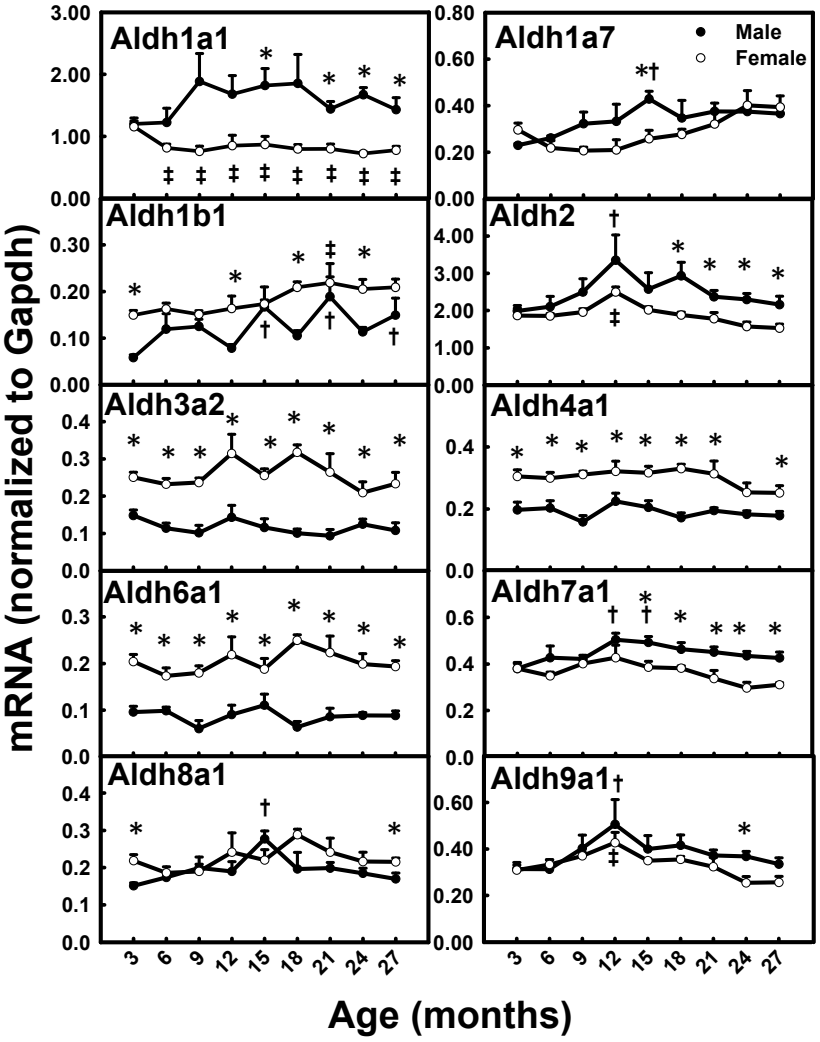
The mRNA profiles of flavin-containing monooxygenases (Fmos) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p < 0.05$). Daggers (†) and double daggers (‡) represent age differences ($p < 0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

Fig. 4.4.



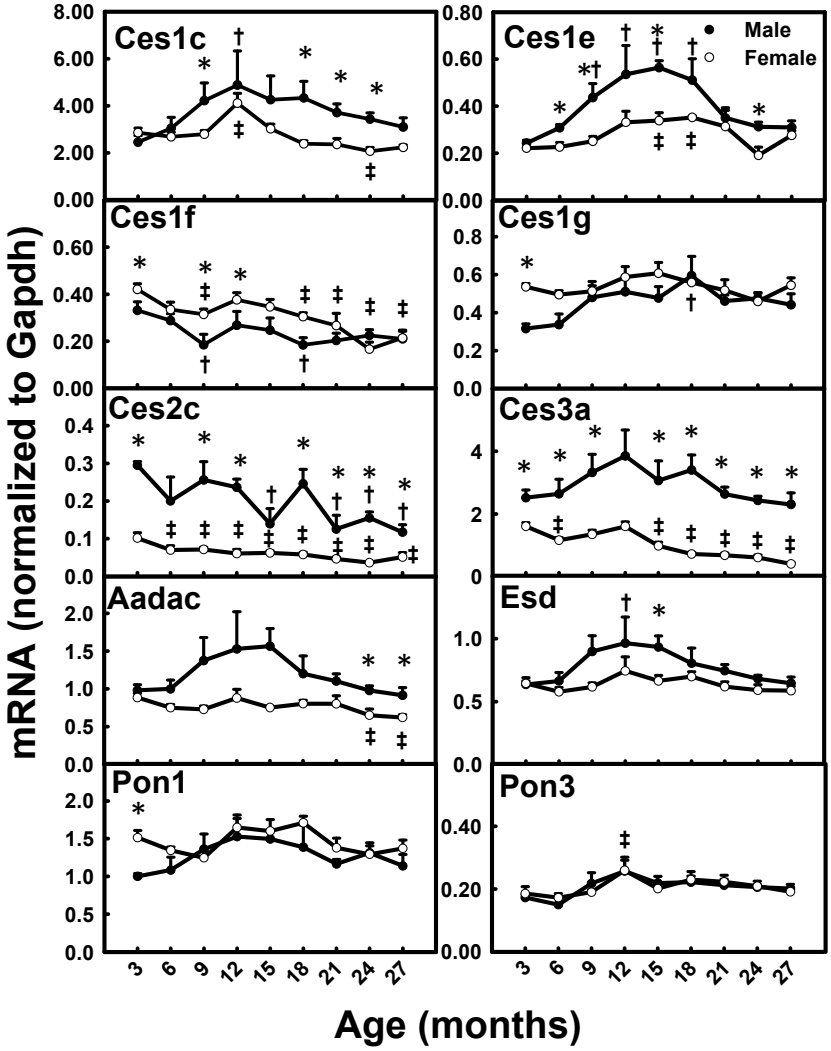
The mRNA profiles of alcohol dehydrogenases (Adhs) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p < 0.05$). Daggers (†) and double daggers (‡) represent age differences ($p < 0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

Fig. 4.5.



The mRNA profiles of aldehyde dehydrogenases (Aldhs) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p < 0.05$). Daggers (†) and double daggers (‡) represent age differences ($p < 0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

Fig. 4.6.



The mRNA profiles of carboxylesterases (Cess) and paraoxonases (Pons) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p < 0.05$). Daggers (†) and double daggers (‡) represent age differences ($p < 0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

4.3.3 Phase−II Xenobiotic Metabolizing Enzymes during Aging.

Sults catalyze the conjugation of xenobiotics with sulfate groups, and the 3'-phosphoadenosine 5'-phosphosulfate synthetase (Papss) catalyzes the synthesis of the sulfate donor for all Sults. In contrast to the changes of mRNA levels of most XPGs during aging, the mRNAs of a number Sults increased with age in mice **(Fig. 4.7)**. Increased mRNAs with age were observed for Sult1a1 (M: 123%; F: 40%), Sult1e1 (M: 9.8-fold; F: 315-fold), Sult2a1/2 (F: 732%), and Papss1 (M: 211%, F: 209%). In contrast, Sult3a1 mRNA decreased 76% in the aged female mice, and Sult5a1 decreased about 50% between 3 and 27 months of age in both genders. The mRNAs of Sult1d1 and Papss2 peaked at 18 months of age in female mice. The mRNA levels of the majority of Sults had marked female-predominant patterns across the lifespan, namely Sult1a1, 1d1, 1e1, 2a1/2, 3a1, and 5a1. In addition, Papss2 mRNA level had a female-predominant pattern.

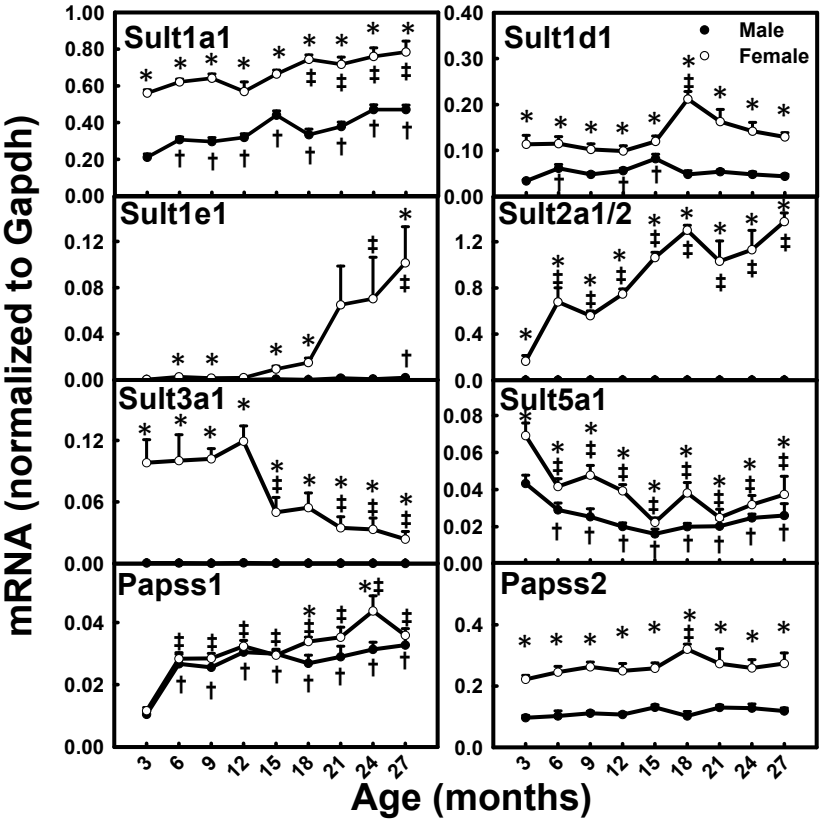
Ugts catalyze the conjugation reaction with glucuronic acids, and UDP-glucose pyrophosphorylase 2 (Ugp2) catalyzes the synthesis of UDP-glucose from glucose-1-phosphate, which is an important precursor for the glucuronidation co-substrate UDP-glucuronic acid. The mRNAs of Ugt2b1 (M: 36%; F: 60%) and Ugt2b35 (M: 30%) decreased between 3 and 27 months of age **(Fig. 4.8)**. Ugt2b36 mRNA also decreased in aging male and female mice. Ugt1a6a mRNA reached peaks at 12 months of age in both genders. The mRNAs of Ugt3a1 and Ugt3a2 reached peaks at 12 months in male mice. Ugp2 mRNA decreased between mid-age and old-age in both genders. The mRNA levels of Ugt1a1, 1a5, 1a9, 2a3, and 2b34 did not change

much with aging. The mRNA levels of some Ugts had female-predominant patterns across the lifespan, namely Ugt1a1, Ugt1a5, Ugt1a9, and Ugt2b34. In contrast, Ugt1a6a, 2b1, and 2b35 had male-predominant pattern.

Gsta1 mRNA increased (M: 183%; F: 124%) between 3 and 27 months of age in both genders **(Fig. 4.9)**. Gstm3 mRNA increased 112% between 3 and 27 months in female mice. A 35-50% decrease in mRNAs between 3 and 27 months was observed for Gstm4 (M), Gstm6 (F), Gstp1 (M), and Gstt2 (M and F). The mRNA of Gstp2 increased 95% between 3 and 27 months in male mice, whereas it decreased 43% in female mice. Mgst3 mRNA increased slightly between 6 and 18 months in both genders. Some of the Gst mRNAs had female-predominant patterns across the lifespan (namely Gstm2, Gstt1, and Mgst3), whereas some had male-predominant patterns (namely Gstm6, Gstp1, and Gstp2). Gstp1 and Gstp2 mRNAs were about 300% higher in male than female mice, whereas Gstt1 and Mgst3 mRNAs were 200% higher in female than male mice.

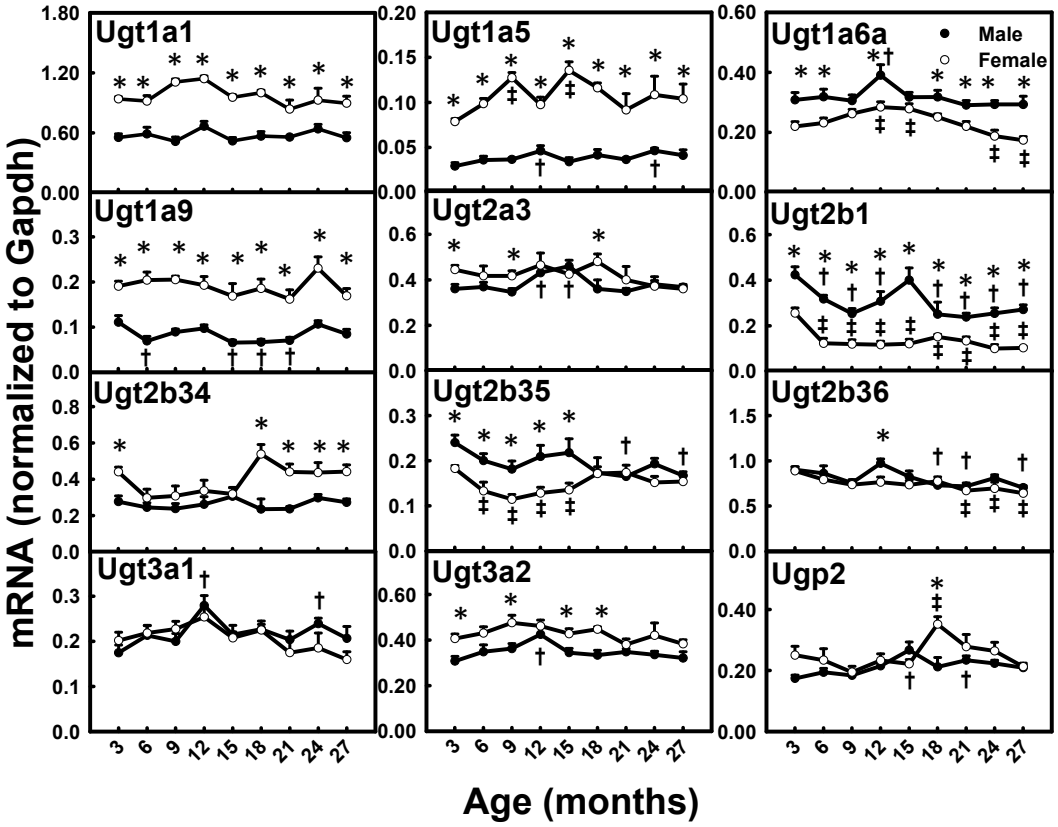
Nat1 mRNA increased (M: 60%; F: 35%) between 3 and 27 months of age, whereas Nat2 mRNA level did not change much with aging **(Fig. 4.10)**. Comt mRNA decreased markedly (93%) between 3 and 27 months of age in female mice, whereas it remained relatively constant in male mice. The mRNAs of Nats did not have marked gender-divergent patterns across the lifespan. Comt mRNA level had a male-predominant pattern in aging mice.

Fig. 4.7.



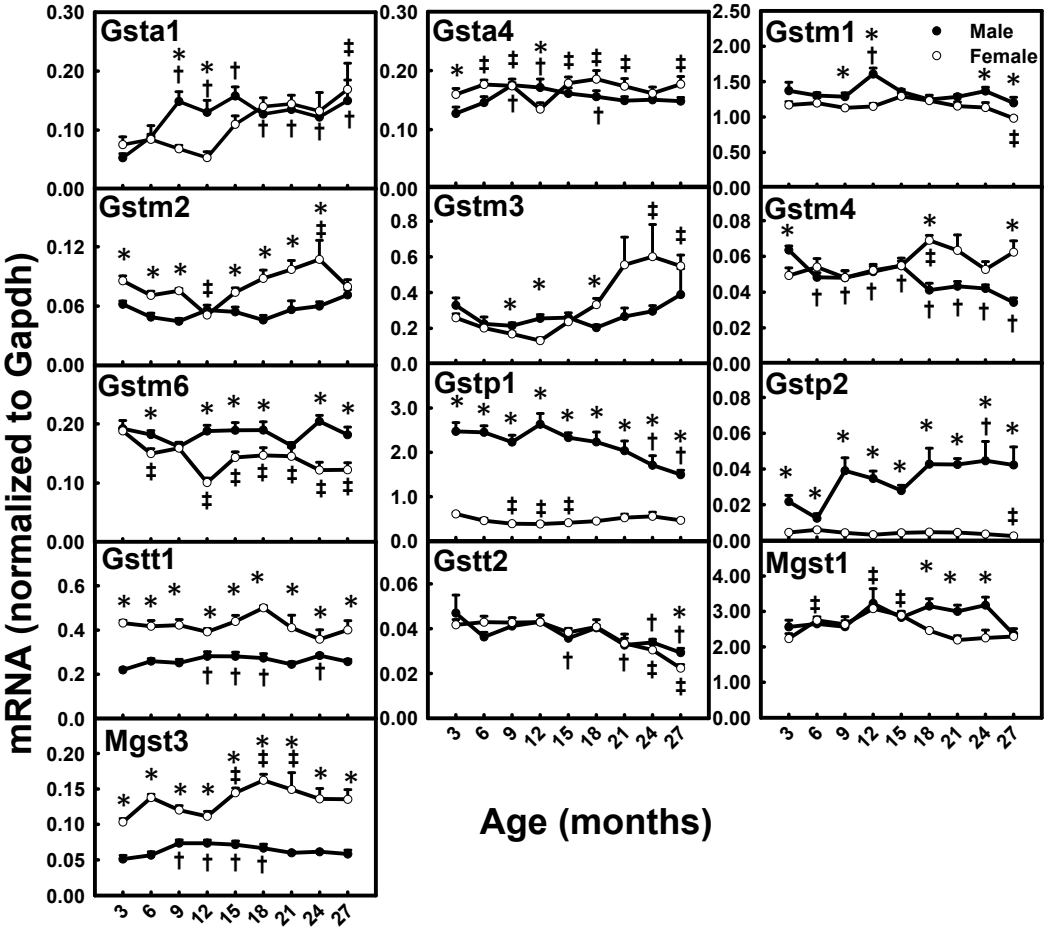
The mRNA profiles of sulfotransferases (Sults) and 3'-phosphoadenosine 5'-phosphosulfate synthetase (Papss) enzymes with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p < 0.05$). Daggers (†) and double daggers (‡) represent age differences ($p < 0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

Fig. 4.8.



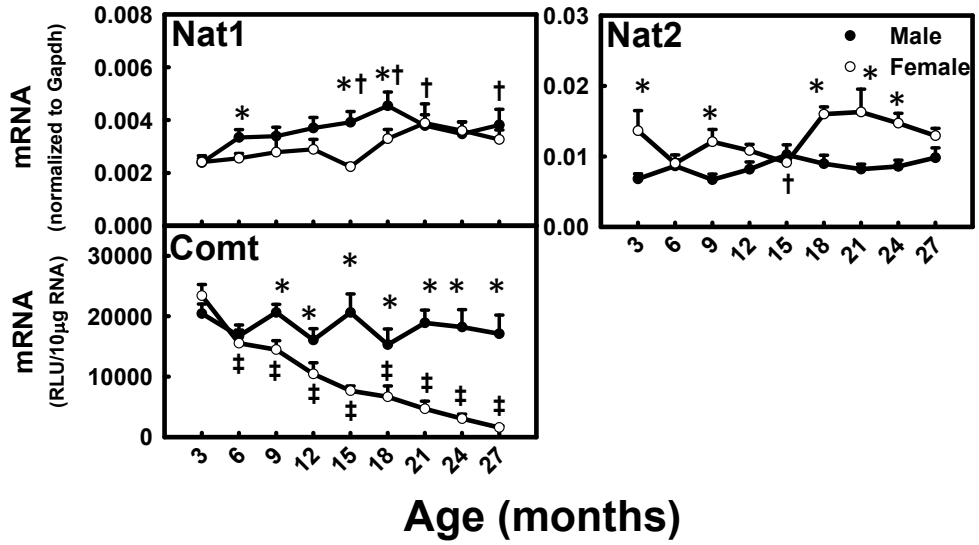
The mRNA profiles of UDP-glucuronosyltransferase (Ugts) and UDP-glucose pyrophosphorylase (Ugp2) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p < 0.05$). Daggers (†) and double daggers (‡) represent age differences ($p < 0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

Fig. 4.9.



The mRNA profiles of glutathione *S*-transferases (Gsts) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p < 0.05$). Daggers (†) and double daggers (‡) represent age differences ($p < 0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

Fig. 4.10.



The mRNA profiles of *N*-acetyltransferases (Nats) and catechol-*O*-methyl transferase (Comt) with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p < 0.05$). Daggers (†) and double daggers (‡) represent age differences ($p < 0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

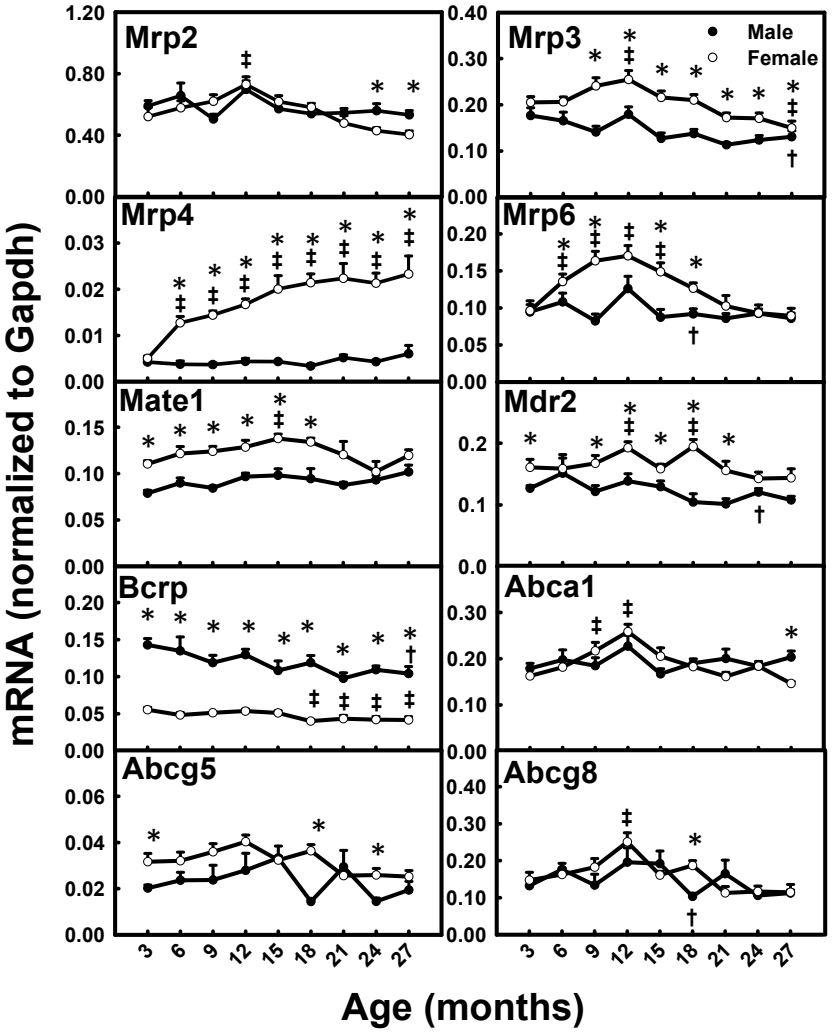
4.3.4 Efflux Transporters during Aging.

Mrp4 mRNA increased 363% between 3 and 27 months of age in female mice, whereas it remained relatively constant in male mice (**Fig. 4.11**). A significant decrease of mRNA between 3 and 27 months was observed in male mice for Mrp3 (26%) and Bcrp (27%). Mrp2, Mrp3, Mrp6, Abca1, and Abcg8 mRNAs peaked at 12 months of age in female mice. The mRNA levels of some efflux transporters had female-predominant mRNA patterns, such as Mrp3, Mrp4, Mate1, and Mdr2. In contrast, Bcrp had a male-predominant mRNA pattern.

4.3.5 Transcription Factors during Aging.

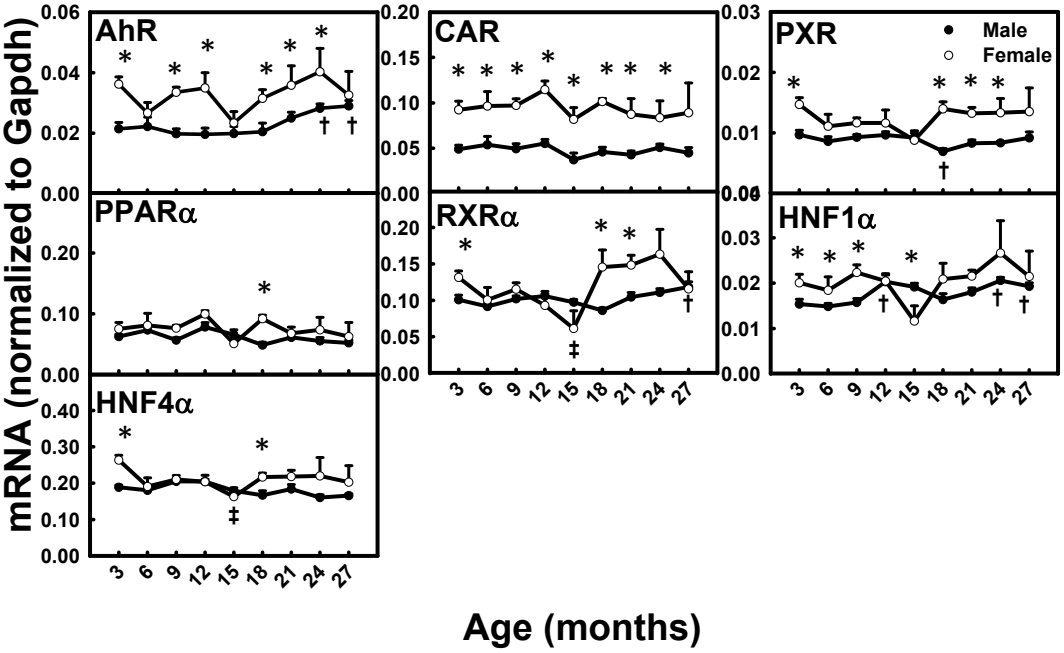
With aging, the mRNA levels of all the transcription factors remained relatively constant (**Fig. 4.12**). The mRNAs of AhR and HNF1α were slightly higher at 24 and 27 months of age than at 3 months in male mice. The mRNA levels of AhR and CAR were female-predominant across the life span. The mRNA levels of remaining transcription factors did not have gender differences.

Fig. 4.11.



The mRNA profiles of efflux transporters with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test (p < 0.05). Daggers (†) and double daggers (‡) represent age differences (p < 0.05) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

Fig. 4.12.

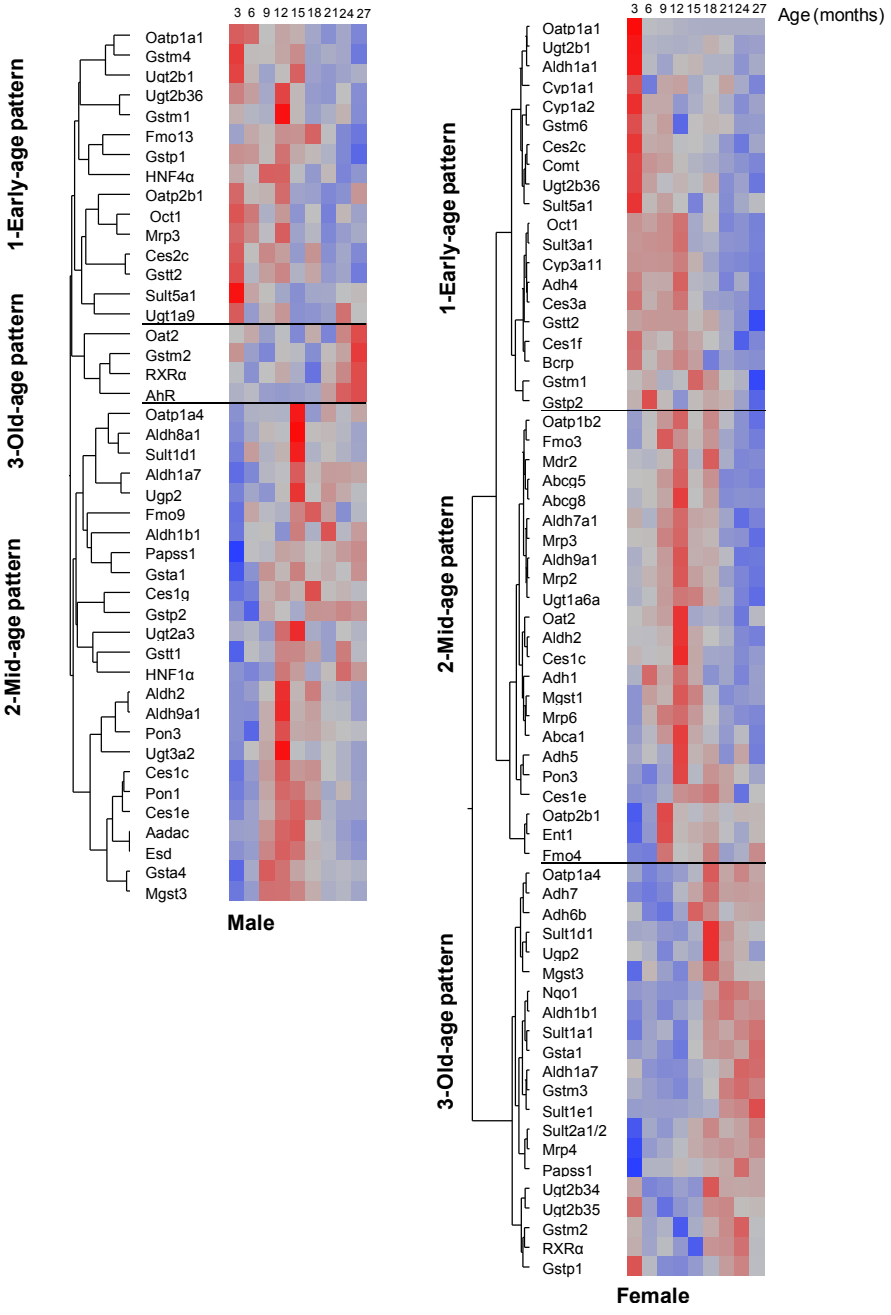


The mRNA profiles of major transcription factors with aging in livers of male and female mice. Asterisks (*) represent gender differences, determined by student t-test ($p < 0.05$). Daggers (†) and double daggers (‡) represent age differences ($p < 0.05$) compared to 3 months of age in male and female mice, respectively, determined by one-way ANOVA, followed by Duncan's post-hoc test.

4.3.6 Hierarchical Cluster Analysis of Age-dependent mRNA Profiles of Xenobiotic Processing Genes.

The mRNA levels of 44% of the 101 XPGs changed with aging in male mice and 63% changed in female mice. The mRNA profiles of these XPGs with age **(Fig. 4.13)** were analyzed by hierarchical cluster analysis, with red color representing relatively high and blue for relatively low mRNAs. The age-dependent changes of mRNA levels of these XPGs could be classified into three clusters for both genders **(Table 4.1)**, namely early-age pattern (highest level observed at 3-9 months of age), mid-age pattern (highest level observed at 12-18 months of age), and old-age pattern (highest level observed at 21-27 months of age). In male mice, mRNAs of 40 XPGs (e.g. Oatp1a1, Ces2c, Gstm4, Gstp1, and Ces1e) were lower in aged mice (over 21 months of age), whereas mRNAs of 4 XPGs (e.g. Oat2 and Gstm2) were higher in aged mice. In female mice, mRNAs of 43 XPGs (e.g. Oatp1a1, Cyp1a2, Ces1f, Sult3a1, Gstt2, Comt, Ent1, Fmo3, and Mrp6) were lower in aged mice, whereas mRNAs of 21 XPGs (e.g. Oatp1a4, Nqo1, Adh7, Sult2a1/2, Gsta1, and Mrp4) were higher in aged mice. In conclusion, the mRNAs of 40% of the XPGs were lower in aged male mice and 43% were lower in aged female mice.

Fig. 4.13.



Hierarchical cluster analysis of the mRNA profiles of XPGs during aging in livers of male and female mice. Clustering analysis results are shown in the dendrogram, with y-axis representing the XPGs that change with aging and x-axis representing the nine age groups of mice (from 3 to 27 months). The red color represents relatively high mRNAs and blue represents relatively low mRNAs. The spectrum of each gene is standardized and specific to the scale of its own mRNA. Therefore, it is not valid to compare the mRNA levels among different genes according to the color.

Table 4.1. Summary of three patterns of age-dependent mRNA changes of XPGs in livers of male and female mice.

Patterns	Male	Female
early-age	15/101*	20/101
mid-age	25/101	23/101
old-age	4/101	21/101

XPGs that don't change with aging: male: 57/101; female: 37/101.

* means the ratio of the number of XPGs in each pattern of age-dependent changes of mRNA to the total number of XPGs.

4.5 Discussion

The present study characterizes comprehensive mRNA profiles of XPGs with aging in livers of mice. Nine groups of mice from 3 to 27 months of age are examined, which gives much more details of age-dependent changes than previous reports (Peng et al., 2005; Mori et al., 2007; Lee et al., 2008). Using both male and female mice, the present study uniquely reveals gender-divergent mRNA profiles of XPGs across the life span.

Many uptake transporters are altered during aging. Oatps are sodium-independent uptake transporters whose substrates are diverse, mainly amphipathic organic compounds, including bile acids, hormones and their conjugates, toxins, and various drugs (Hagenbuch and Gui, 2008). The mRNA of liver-enriched Oatp1a1 decreases more than 50% in aged males, and surprisingly decreases almost 100% in aged females **(Fig. 4.1)**. The mRNA of liver-specific Oatp1b2 decreases from 12 to 27 months of age in females. The mRNA of organic cation uptake transporter Oct1 decreases during aging in both genders. Taken together, liver appears to remove xenobiotics from the blood more slowly in the elderly.

The phase-I enzymes that are altered most during aging are Cyp and Ces families. Cyps are heme-containing enzymes, which catalyze monooxygenase reactions. The Cyp1, Cyp2, and Cyp3 families are the Cyps primarily involved in the metabolism of drugs, xenobiotics, and steroids (Monostory and Dvorak, 2011). Most of the Cyps quantified in the present study have an age-dependent decrease in mRNAs, such as Cyp1a1 (F) and Cyp1a2 (M and F), as well as Cyp3a11 (F) to a lesser degree **(Fig. 4.**

2.). This suggests a possible mechanism for decreased metabolism of some xenobiotics in the elderly. Cess catalyze the hydrolysis of many clinically useful drugs with ester moieties, resulting in mainly inactivation of drugs (such as heroin, cocaine, and flumazenil) as well as the activation of prodrugs (such as anticancer drugs CPT-11 and capecitabine) (Redinbo and Potter, 2005). The nomenclature of mouse Cess in the present study was reported previously (Holmes et al., 2010). The decreased mRNAs of Cess1f, 2c, and 3a during aging in females **(Fig. 4.6)** indicates it may take longer for the anticancer prodrugs to take effect in the elderly, and the ester drugs may have longer half-life in the elderly. For other phase-I enzymes, such as Fmos **(Fig. 4.3)**, Adhs **(Fig. 4.4)**, Aldhs **(Fig. 4.5)**, and Pons **(Fig. 4.6)**, aging has little effect on their mRNA levels.

Aging has profound effects on altering the mRNA levels of phase-II enzymes. All of the Sults quantified in the present study remain female-predominant during aging. The mRNAs of Sult1a1 and Papss1 increase during aging in both genders. Sult1e1 mRNA increases 9.8-fold in males and 315-fold in females between 3 and 27 months of age **(Fig. 4.7)**. Sult1e1 sulfonates a variety of estrogens (Falany et al., 1995), and is the predominant determinant of the ratio of the active unconjugated estrogen to the inactive estrogen sulfate. In addition to the aging-induced decline of ovarian function and female sex hormone secretion, the increased Sult1e1 and possibly increased inactivation of estrogens might also contribute to decreased biologically active estrogens in aged females. The mRNA level of Sult2a1/2 is abundant in females, but extremely low in males at young adulthood (3-month old), which is consistent with our previous publication (Alnouti and Klaassen, 2006). The DHEA-sulfotransferase Sult2A is known

as a rat senescence marker protein, the expression of which is increased in aged male rats (Echchgadda et al., 2004). The present study shows that Sult2a1/2 mRNA increases 732% in aged female, but remains at extremely low levels in males during aging **(Fig. 4.7)**. This discrepancy may result from species differences. Additional work is necessary to definitely determine the mechanisms underlying the age- and sex-dependent regulation of Sult2a1/2 in mice. In contrast to the elevated mRNAs of Sult1a1, 1e1, and 2a1/2, Sult3a1 mRNA decreases 76% in the aged female mice. Sult3a1 is the only Sult isozyme that catalyzes *N*-sulfonation, rather than *O*-sulfonation of amines such as phenyltetrahydropyridine, aniline, 4-chloroaniline, 2-naphthylamine, and desipramine (Yoshinari et al., 1998). 4-Chloroaniline is carcinogenic in male rats and mice (Chhabra et al., 1991), mainly because of the toxic intermediate *N*-phenylhydroxylamine. The C-hydroxylated product of 4-chloroaniline is sulfated and ready for excretion. The decreased mRNA of Sult3a1 after 12 months of age in male mice **(Fig. 4.7)** suggests that the detoxification of 4-chloroaniline might decline with age, which could favor cancer development induced by 4-chloroaniline.

The mRNAs of most Ugts remain relatively constant with aging **(Fig. 4.8)**. However, Ugt2b1 mRNA decreases between 3 and 27 months of age in both genders. A previous study (Buckley and Klaassen, 2009) reported that the regulation of Ugt2b1 expression in mouse liver was attributed to male-pattern growth hormone secretion. This may explain the dramatic decrease of Ugt2b1 expression for male mice with age, and less dramatic for females.

Gsts catalyze the biotransformation and disposition of a wide range of chemical carcinogens, therapeutic drugs, the products of oxidative stress, and steroid hormones such as Δ5-androstenedione (Johansson and Mannervik, 2001). Gstp1 mRNA decreases with age **(Fig. 4.9)**. Gstp2 mRNA increases with age, but is at a much lower level than Gstp1. Gstp catalyzes the glutathione conjugation of cisplatin as a detoxification pathway (Townsend et al., 2009). The decrease in Gstp1 mRNA with aging in the present study provides a possible mechanism of changes in cisplatin toxicity during aging.

The Comt catalyzes the methylation of catecholamine neurotransmitters, L-DOPA, catecholestrogens (2- and 4-hydroxylated estrogen), as well as drugs, such as carbidopa and dobutamine. The highest Comt activity has been found in liver and kidney. Comt activity is also detected in several glands, muscle, adipose, blood cells and other tissues. One important function of Comt is to detoxify catecholestrogens, which appears to be important in initiating some estrogen-dependent cancers, possibly by generating reactive oxygen species and subsequent DNA damage (Weisz et al., 1998). The decreased Comt with aging in females **(Fig. 4.10)** suggest the detoxification of catecholestrogens in livers might decrease in aged females.

Most efflux transporters are altered during aging. The mRNAs of Mrp3 and Bcrp decrease during aging in both genders **(Fig. 4.11)**. Mrp3 is important in transporting bilirubin glucuronides and bile acids from liver into blood, whereas Bcrp is important in liver for transporting sulfate and glucuronide conjugates of xenobiotics into bile. The decreased expression of Mrp3 and Bcrp with age suggests that glucuronide conjugates

of xenobiotics as well as bilirubin might accumulate in hepatocytes with age. Interestingly, Mrp4 mRNA increases between 3 and 27 months of age in female mice **(Fig. 4.11)**. A previous study shows higher mRNA of Mrp4 in female kidneys is due to repression by both 5alpha-dihydroxytesteosterone and male-pattern growth hormone secretion in males (Maher et al., 2006). Hypothesizing that similar mechanisms of hormonal regulation of female-predominant expression of Mrp4 exist in liver, the elevation in Mrp4 mRNA during aging possibly results from the decline of sex hormones and growth hormones with senescence.

The mRNAs of key transcription factors quantified in the study remained relatively constant during aging **(Fig. 4.12)**. No apparent correlation between the mRNA profiles of XPG and transcription factors were observed across aging process **(Fig. 4.13)**.

All of the data collected in the present study regarding the expression of XPGs are at the mRNA level. One needs to be cautious when interpreting the data, because mRNA levels don't always correlate with protein levels and protein activities. According to previous reports that the decreased protein contents (Peng et al., 2005; Mori et al., 2007) as well as enzyme activities (Warrington et al., 2004) of many phase-I and phase-II enzymes are regulated at the transcriptional level, the results from this study will provide a good indication for most enzymes and transporters. For transporters, it is especially difficult to quantify protein and activity of transporters, due to the lack of specific antibodies as well as specific substrates for activity assays.

In summary, the current study investigates the effect of aging on the mRNA levels of 101 xenobiotic processing genes (XPGs) in livers of male and female mice. Gender differences across the lifespan (significant at five ages or more) are observed for 52 XPGs, including 15 male-predominant (e.g. Oatp1a1, Cyp3a11, Ugt1a6a, Comt, and Bcrp) and 37 female-predominant genes (e.g. Oatp1a4, Cyp2b10, Sult1a1, Ugt1a1, and Mrp3). The mRNA levels of 44% of the XPGs change with aging in male mice and 63% change in female mice. In male mice, mRNAs of 40 XPGs (e.g. Oatp1a1, Ces2c, Gstm4, Gstp1, and Ces1e) are lower in aged mice (over 21 months of age), whereas mRNAs of 4 XPGs (e.g. Oat2 and Gstm2) are higher in aged mice. In female mice, mRNAs of 43 XPGs (e.g. Oatp1a1, Cyp1a2, Ces1f, Sult3a1, Gstt2, Comt, Ent1, Fmo3, and Mrp6) are lower in aged mice, whereas mRNAs of 21 XPGs (e.g. Oatp1a4, Nqo1, Adh7, Sult2a1/2, Gsta1, and Mrp4) are higher in aged mice. In conclusion, 51% of the 101 XPGs have gender differences in liver mRNA levels across the lifespan of mice, and the mRNAs of 40% of the XPGs are lower in aged male mice and 43% are lower in aged female mice. Considering the role of XPGs in detoxification and elimination of drugs and environmental chemicals, the present results may improve the interpretation of long-term toxicity studies in old animals, and aid in determining the effective and safe dose for the elderly.

Chapter 5: REGULATION OF BILE ACID HOMEOSTASIS DURING AGING OF MICE

This study was published as Fu et al., 2012. PLoS One, 7, e32551.

5.1 Abstract

Aging is a physiological process with a progressive decline of adaptation and functional capacity of the body. BAs have been recognized as signaling molecules regulating the homeostasis of glucose, lipid, and energy. The current study characterizes the age-related changes of individual BA concentrations by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) in serum and liver of male and female C57BL/6 mice from 3 to 27 months of age. Total BA concentrations in serum increased 340% from 3 to 27 months in female mice, whereas they remained relatively constant with age in male mice. During aging, male and female mice shared the following changes: (1) BA concentrations in liver remained relatively constant; (2) the proportions of beta-muricholic acid (βMCA) increased and deoxycholic acid (DCA) decreased between 3 and 27 months in serum and liver; and (3) total BAs in serum and liver became more hydrophilic between 3 and 27 months. In female mice, (1) the mRNAs of hepatic BA uptake transporters, the Na^+/taurocholate cotransporting polypeptide (Ntcp) and the organic anion transporting polypeptide 1b2 (Oatp1b2), decreased after 12 months, and similar trends were observed for their proteins; (2) the mRNA of the rate-limiting enzyme for BA synthesis, cholesterol 7α-hydroxylase (Cyp7a1), increased from 3 to 9 months and remained high thereafter. However, in male mice, Ntcp, Oatp1b2, and Cyp7a1 mRNA levels remained relatively constant with age. In summary, the current study shows gender-divergent profiles of BA concentrations and composition in serum and liver of mice during aging, which is

likely due to the gender-divergent expression of BA transporters Ntcp and Oatp1b2 as well as the synthetic enzyme Cyp7a1.

5.2 Introduction

Elderly people have an increased incidence of various age-related diseases, including liver and gastrointestinal diseases. The prevalence of chronic liver disease increases in the elderly, such as alcoholic liver disease, non-alcoholic fatty liver disease, viral hepatitis C, as well as hepatocellular carcinoma (Frith et al., 2009). In addition, the risk of stomach cancer increases with age, and more than 90% of colon cancers were found in people over 50-years of age.

In the enterohepatic system, BAs play multifaceted physiological functions. Apart from their well-known roles for dietary lipid absorption and cholesterol homeostasis, BAs are increasingly appreciated as complex metabolic signaling molecules (Li and Chiang, 2012), regulating glucose, lipid, and energy metabolism. In humans, up to 95% of the BAs are efficiently recycled daily through the "enterohepatic circulation" (EHC), and only 5% are newly synthesized.

BAs can regulate their own homeostasis (Zhang and Klaassen, 2010; Song et al., 2011). BAs activate their nuclear receptor FXR in liver, which transactivates small heterodimer partner (SHP). SHP subsequently forms inactive heterodimer with liver related homologue-1 (LRH-1) (Goodwin et al., 2000), resulting in decreased transcription of Cyp7a1. In addition, BAs also activate FXR in the intestine, which induces fibroblast growth factor 15 (Fgf15), an intestinal hormone that travels through the circulation to the liver and down-regulates Cyp7a1 transcription (Inagaki et al., 2005; Kim et al., 2007).

Limited information is available on the effect of aging on BA homeostasis (Uchida et al., 1978; Bertolotti et al., 1993; Salemans et al., 1993a). Bertolotti *et al.* studied the effect of aging on cholesterol 7 alpha-hydroxylation, which is the rate-limiting step of bile acid synthesis. Among the 28 patients of 34-83 year old of both sexes, they found a reduced rate of conversion of cholesterol to BAs during aging, particularly in females, evidenced by a tritium release assay (Bertolotti et al., 1993). It was also reported in the same year that postprandial serum levels of conjugated cholic, chenodeoxycholic, and deoxycholic acid were increased more in the younger subjects, whereas postprandial serum levels of unconjugated BAs didn't differ between younger and elderly age groups. This indicates that conjugated BAs are reabsorbed less effectively in the elderly, which may lead to increased risk of colorectal cancer (Salemans et al., 1993a).

In a more recent study, Amador-Noguez *et al.* reported increased expression of xenobiotic metabolizing genes in livers of long-lived lit/lit mice, and concluded that FXR is the only nuclear receptor responsible for the up-regulation of gene expression among the three potential regulators studied. Because FXR is known to be activated by BAs and it increases the mRNAs of BA metabolizing enzymes, they administered cholic acid to wild-type mice and were able to reproduce the changes in drug metabolizing gene expression seen in lit/lit mice (Amador-Noguez et al., 2007). Therefore, a hypothetical model of aging-regulatory systems in lit/lit mice characterized by decreased IGF-1 and increased BAs by blocking growth hormone signaling that retard aging was proposed (Gems, 2007). This paper is novel because it indicates the possible role of BAs as contributors to longevity. A hormesis effect was proposed as a possible explanation to

the pro-longevity effect of low levels of BAs which are toxic at high levels (Song et al., 2011). A similar concept was proved in C. elegans that transient heat stress could increase their lifespan (Cypser et al., 2006). In addition, chemical genetic screen identified lithocholic acid as an anti-aging compound in yeast in a TOR-independent manner (Goldberg et al., 2010). Moreover, 3-keto BA-like steroids, called dafachronic acids, increase the life span of *C. elegans*, through DAF-12 (a homolog of FXR) (Held et al, 2006; Gerisch et al., 2007). Although the whole field of BA homeostasis during aging still needs a lot of research, the idea of BAs contributing to the longevity is very intriguing (Ferbeyre, 2010b; Roux and Chartrand, 2010).

The current study aims to describe the effect of aging on BA composition and concentration, and determine the molecular mechanism with respect to the expression of genes involved in BA homeostasis in mice. To address these questions, both male and female mice at nine ages, from 3- to 27-months old, were used for serum and tissue collection for BA, mRNA, and protein quantification. Substantial information on the changes of BA concentrations and composition in male and female mice during aging was obtained, as well as possible regulatory mechanisms for these changes.

5.3 Results

5.3.1 Total BA Concentrations in Serum during Aging.

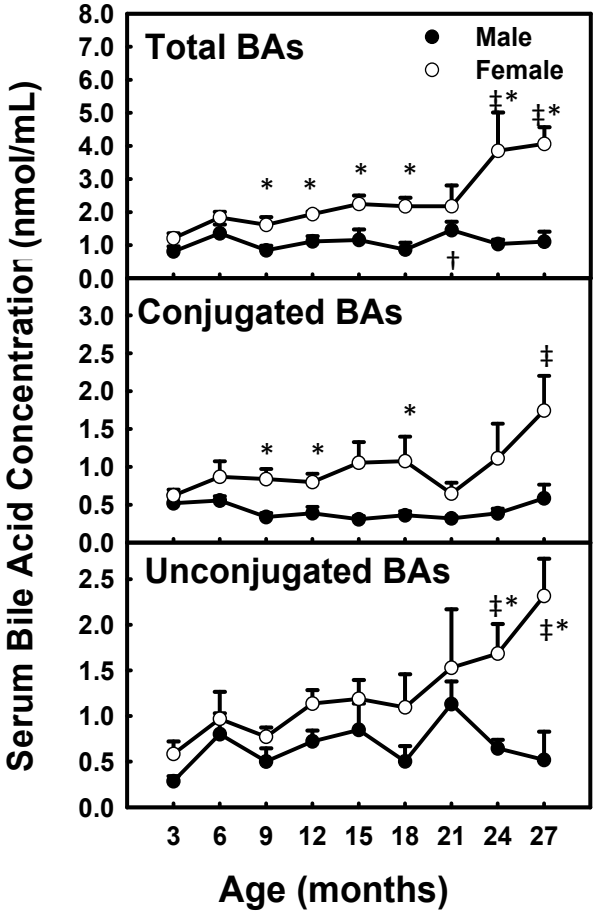
Total BA concentrations in serum remained relatively constant in male mice, whereas in female mice, they increased 340% from 3 to 27 months of age, due to an increase in both conjugated (280%) and unconjugated (400%) BAs **(Fig. 5.1)**. Female mice had higher total BA concentrations than male mice from 9 (190%) to 27 (370%) months.

5.3.2 Conjugated BAs in Serum during Aging.

In male mice, the concentrations of most conjugated BAs (T-CA, T-αMCA, T-βMCA, T-UDCA, and T-HDCA) remained relatively constant during aging **(Fig. 5.2A)**. In female mice, the concentrations of three BAs increased between 3 and 27 months, namely T-βMCA (610%), T-UDCA (470%), and T-ωMCA (260%). However, the concentrations of T-CA, T-αMCA, T-DCA, T-MDCA, and T-HDCA remained relatively constant with age.

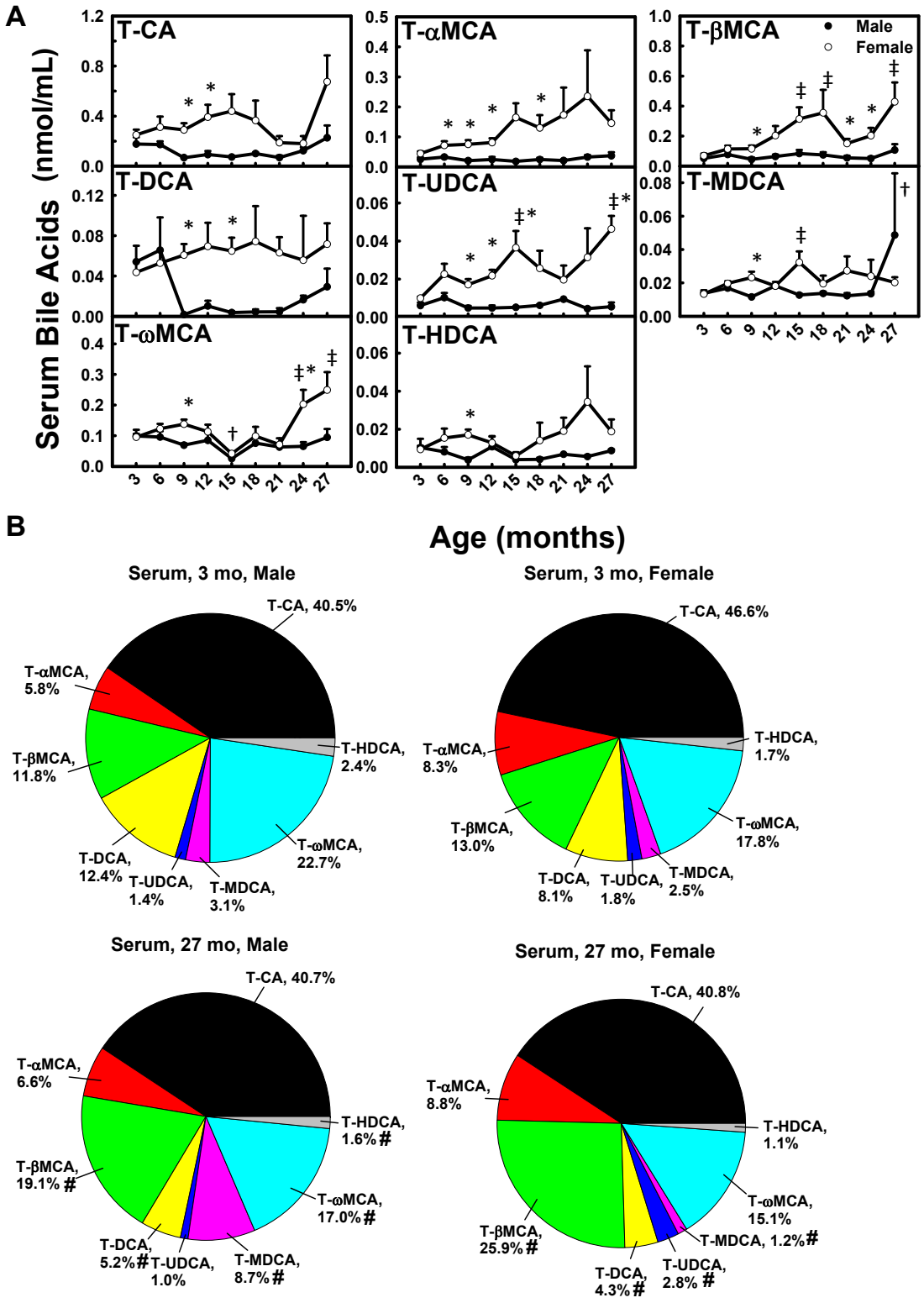
The composition of conjugated BAs in serum changed during aging **(Fig. 5.2B)**. In male mice, the proportions of T-βMCA (11.8%→19.1%) and T-MDCA (3.1%→8.7%) increased, whereas T-DCA (12.4%→5.2%) and T-HDCA (2.4%→1.6%) decreased between 3 and 27 months of age. In female mice, the proportions of T-βMCA (13.0%→25.9%) and T-UDCA (1.8%→2.8%) increased, whereas T-MDCA (2.5%→1.2%) and T-DCA (8.1%→4.3%) decreased between 3 and 27 months.

Fig. 5.1.



Total BA concentrations in serum during aging of male and female mice. Data are presented as means ± SEM of 5-7 mice. Daggers (†) represent statistically significant difference from the value at 3 months of age during aging of male mice. Double daggers (‡) represent statistically significant difference from the value at 3 months of age during aging of female mice. Age-dependent differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. Asterisks (*) represent statistically significant difference between male and female mice at respective ages during aging ($p < 0.05$), by Student's *t*-test.

Fig. 5.2.


A
Serum Bile Acids (nmol/mL)
T-CA
T-αMCA
T-βMCA
T-DCA
T-UDCA
T-MDCA
T-ωMCA
T-HDCA
Male
Female
Age (months)
B
Serum, 3 mo, Male
T-CA, 40.5%
T-αMCA, 5.8%
T-βMCA, 11.8%
T-HDCA, 2.4%
T-ωMCA, 22.7%
T-DCA, 12.4%
T-UDCA, 1.4%
T-MDCA, 3.1%
Serum, 3 mo, Female
T-CA, 46.6%
T-αMCA, 8.3%
T-HDCA, 1.7%
T-βMCA, 13.0%
T-ωMCA, 17.8%
T-DCA, 8.1%
T-UDCA, 1.8%
T-MDCA, 2.5%
Serum, 27 mo, Male
T-CA, 40.7%
T-αMCA, 6.6%
T-HDCA, 1.6%#
T-βMCA, 19.1%#
T-ωMCA, 17.0%#
T-DCA, 5.2%#
T-UDCA, 1.0%
T-MDCA, 8.7%#
Serum, 27 mo, Female
T-CA, 40.8%
T-αMCA, 8.8%
T-HDCA, 1.1%
T-βMCA, 25.9%#
T-ωMCA, 15.1%
T-MDCA, 1.2%#
T-DCA, 4.3%#
T-UDCA, 2.8%#

Continued to the following page.

Fig. 5.2. (continued)

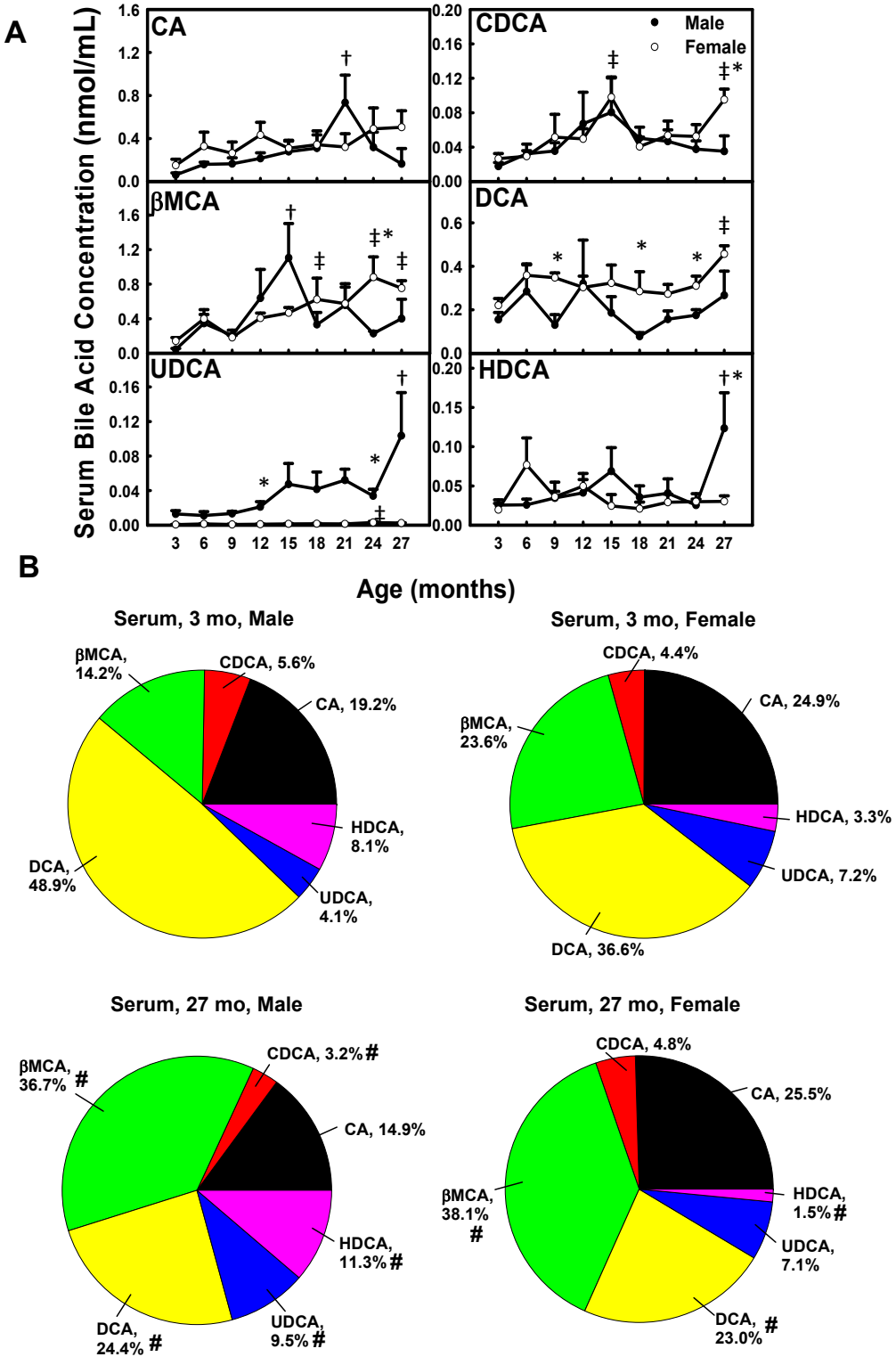
Concentrations of conjugated BAs in serum during aging of male and female mice. (A) Concentrations of individual conjugated BAs in serum during aging and (B) proportions of them in all conjugated BAs in serum at 3 and 27 months of age in male and female mice. Data are presented as means ± SEM of 5-7 mice. Daggers (†) represent statistically significant difference from the value at 3 months of age during aging of male mice. Double daggers (‡) represent significant difference from the value at 3 months of age during aging of female mice. Age-dependent differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. Asterisks (*) represent statistically significant difference between male and female mice at respective ages during aging ($p < 0.05$), by Student's *t*-test. In panel B, pound signs (#) represent differences of BA proportions between 3 and 27 months.

5.3.3 Unconjugated BAs in Serum during Aging.

In male mice, the concentration of UDCA increased 800% from 3 to 27 months of age **(Fig. 5.3A)**. CA increased (12.1-fold) from 3 to 21 months and decreased thereafter. Similarly, βMCA increased 24.6-fold from 3 to 15 months and decreased thereafter, and HDCA increased 480% from 3 to 27 months and decreased thereafter. CDCA and DCA remained relatively constant with age. In female mice, gradual age-dependent increase in concentrations of βMCA (530%) and DCA (210%) was observed between 3 and 27 months. CDCA was higher at 15 (370%) and 27 (360%) months than at 3 months of age. UDCA was 430% higher at 24 than 3 months. CA and HDCA remained relatively constant with age.

The composition of unconjugated BAs in serum also changed during aging **(Fig. 5.3B)**. In male mice, the proportions of βMCA (14.2%→36.7%), UDCA (4.1%→9.5%), and HDCA (8.1%→11.3%) increased, whereas CDCA (5.6%→3.2%) and DCA (48.9%→24.4%) decreased between 3 and 27 months of age. In female mice, the proportion of βMCA (23.6%→38.1%) increased, whereas DCA (36.6%→23.0%) and HDCA (3.3%→1.5%) decreased between 3 and 27 months.

Fig. 5.3.


A
Serum Bile Acid Concentration (nmol/mL)
CA
CDCA
βMCA
DCA
UDCA
HDCA
Male
Female
Age (months)
B
Serum, 3 mo, Male
βMCA, 14.2%
CDCA, 5.6%
CA, 19.2%
HDCA, 8.1%
UDCA, 4.1%
DCA, 48.9%
Serum, 3 mo, Female
CDCA, 4.4%
CA, 24.9%
βMCA, 23.6%
HDCA, 3.3%
UDCA, 7.2%
DCA, 36.6%
Serum, 27 mo, Male
βMCA, 36.7% #
CDCA, 3.2% #
CA, 14.9%
HDCA, 11.3% #
UDCA, 9.5% #
DCA, 24.4% #
Serum, 27 mo, Female
CDCA, 4.8%
CA, 25.5%
βMCA, 38.1% #
HDCA, 1.5% #
UDCA, 7.1%
DCA, # 23.0%

Continued to the following page.
Fig. 5.3. (continued)

Concentrations of unconjugated BAs in serum during aging of male and female mice. (A) Concentrations of individual unconjugated BAs in serum during aging and (B) proportions of them in all unconjugated BAs in serum at 3 and 27 months of age in male and female mice. Data are presented as means ± SEM of 5-7 mice. Daggers (†) represent statistically significant difference from the value at 3 months of age during aging of male mice. Double daggers (‡) represent significant difference from the value at 3 months of age during aging of female mice. Age-dependent differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. Asterisks (*) represent statistically significant difference between male and female mice at respective ages during aging ($p < 0.05$), by Student's *t*-test. In panel B, pound signs (#) represent differences of BA proportions between 3 and 27 months.

5.3.4 Total BA Concentrations in Liver during Aging.

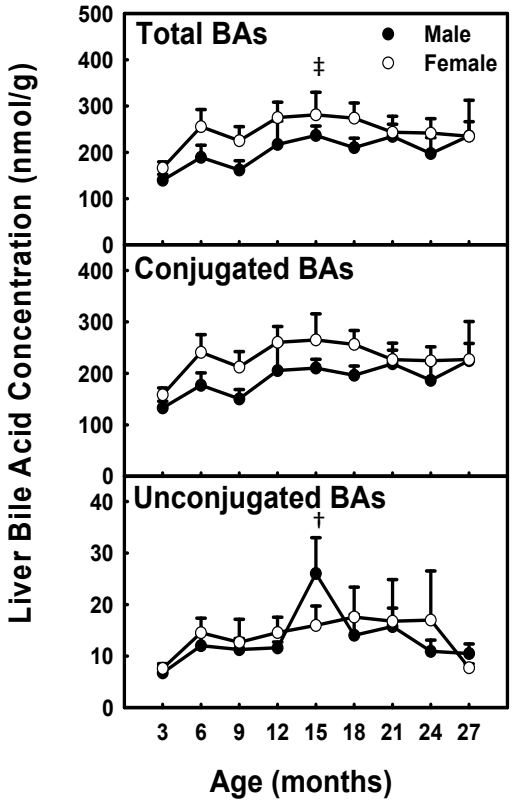
The concentrations of total, conjugated, and unconjugated BAs in liver remained relatively constant with age, except a small increase (170%) of total BAs at 15 months of age in female mice, and a small increase (390%) of unconjugated BAs at 15 months in male mice **(Fig. 5.4)**.

5.3.5 Conjugated BAs in Liver during Aging.

In male mice, the concentration of T-α+βMCA gradually increased (350%), whereas T-DCA gradually decreased (65.8%) between 3 and 27 months of age **(Fig. 5.5A)**. T-CA, T-CDCA, T-LCA, T-UDCA, T-MDCA, T-ωMCA, and T-HDCA remained relatively constant with age. In female mice, the concentrations of T-CDCA, T-α+βMCA, T-UDCA, and T-MDCA doubled between 3 and 15 months, and decreased thereafter. Similarly, T-LCA doubled between 3 and 9 months and decreased thereafter. T-CA, T-DCA, T-ωMCA, and T-HDCA remained relatively constant with age.

The composition of conjugated BAs in liver changed during aging **(Fig. 5.5B)**. In male mice, the proportion of T-α+βMCA (20.2%→42.1%) increased, whereas T-DCA (11.0%→2.2%), T-LCA (0.5%→0.1%), and T-ωMCA (16.6%→9.5%) decreased between 3 and 27 months of age. In female mice, the proportion of T-α+βMCA (29.6%→35.7%) increased, whereas T-DCA (6.2%→3.7%), T-LCA (0.3%→0.1%), T-MDCA (1.4%→0.9%), and T-HDCA (1.7%→0.9%) decreased between 3 and 27 months.

Fig. 5.4.



Total BA concentrations in liver during aging of male and female mice. Data are presented as means ± SEM of 5-7 mice. Daggers (†) represent statistically significant difference from the value at 3 months of age during aging of male mice. Double daggers (‡) represent significant difference from the value at 3 months of age during aging of female mice. Age-dependent differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. Asterisks (*) represent statistically significant difference between male and female mice at respective ages during aging ($p < 0.05$), by Student's *t*-test.

Fig. 5.5.

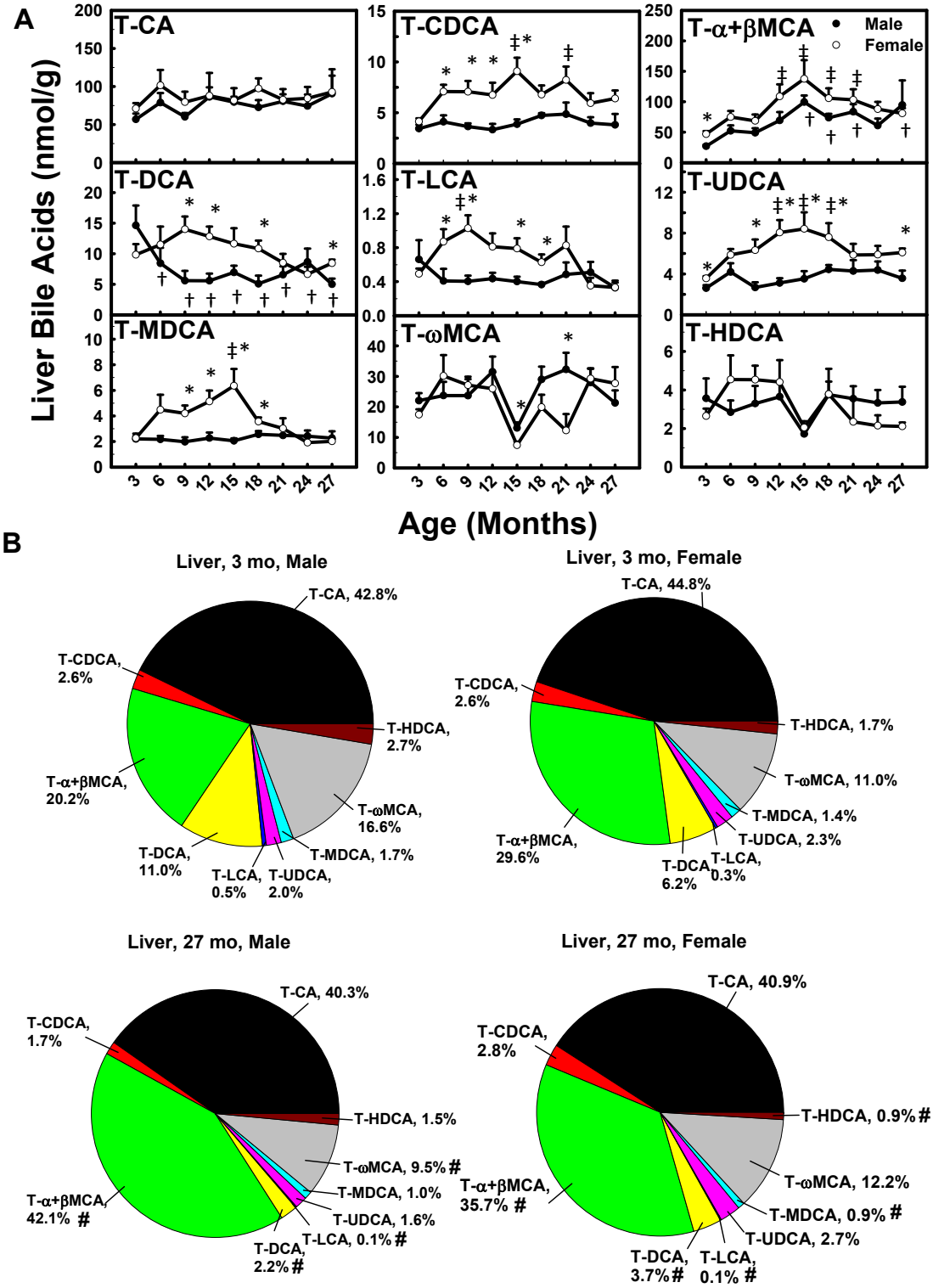


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Fig. 5.5. (continued)

Concentrations of conjugated BAs in liver during aging of male and female mice. (A) Concentrations of individual conjugated BAs in liver during aging and (B) proportions of them in all conjugated BAs in liver at 3 and 27 months of age in male and female mice. Data are presented as means ± SEM of 5-7 mice. Daggers (†) represent statistically significant difference from the value at 3 months of age during aging of male mice. Double daggers (‡) represent significant difference from the value at 3 months of age during aging of female mice. Age-dependent differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. Asterisks (*) represent statistically significant difference between male and female mice at respective ages during aging ($p < 0.05$), by Student's *t*-test. In panel B, pound signs (#) represent differences of BA proportions between 3 and 27 months.

5.3.6 Unconjugated BAs in Liver during Aging.

In male mice, The concentration of βMCA increased (530%) from 3 to 15 months and decreased 50.8% thereafter **(Fig. 5.6A)**. In female mice, CA was 390% higher at 18 than 3 months, and ωMCA increased (170%) between 3 and 15 months and decreased 52.1% thereafter. CDCA, αMCA, DCA, LCA, UDCA, and HDCA remained relatively constant with age in both genders.

The composition of unconjugated BAs in liver changed during aging **(Fig. 5.6B)**. In male mice, the proportion of βMCA (38.9%→66.4%) increased, whereas CA (11.2%→7.8%), DCA (2.5%→0.6%), LCA (0.2%→0.1%), UDCA (1.3%→0.9%), MDCA (2.2%→1.4%), ωMCA (32.3%→14.8%), and HDCA (2.7%→1.6%) decreased between 3 and 27 months. In female mice, αMCA (6.4%→4.3%), DCA (1.9%→1.2%), LCA (0.2%→0.05%), MDCA (1.9%→0.7%), and HDCA (1.5%→0.6%) decreased between 3 and 27 months.

Fig. 5.6.

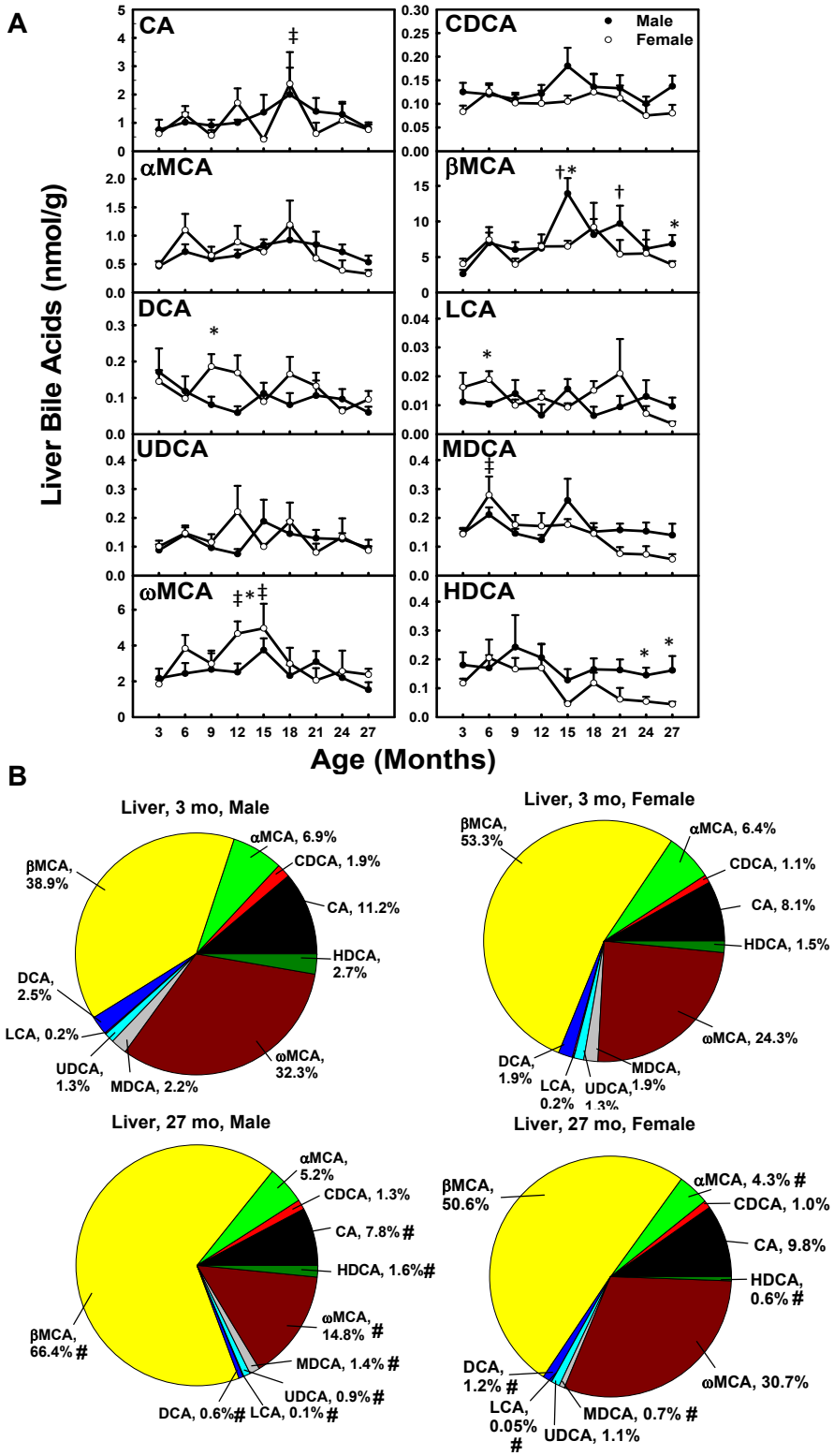

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Fig. 5.6. (continued)

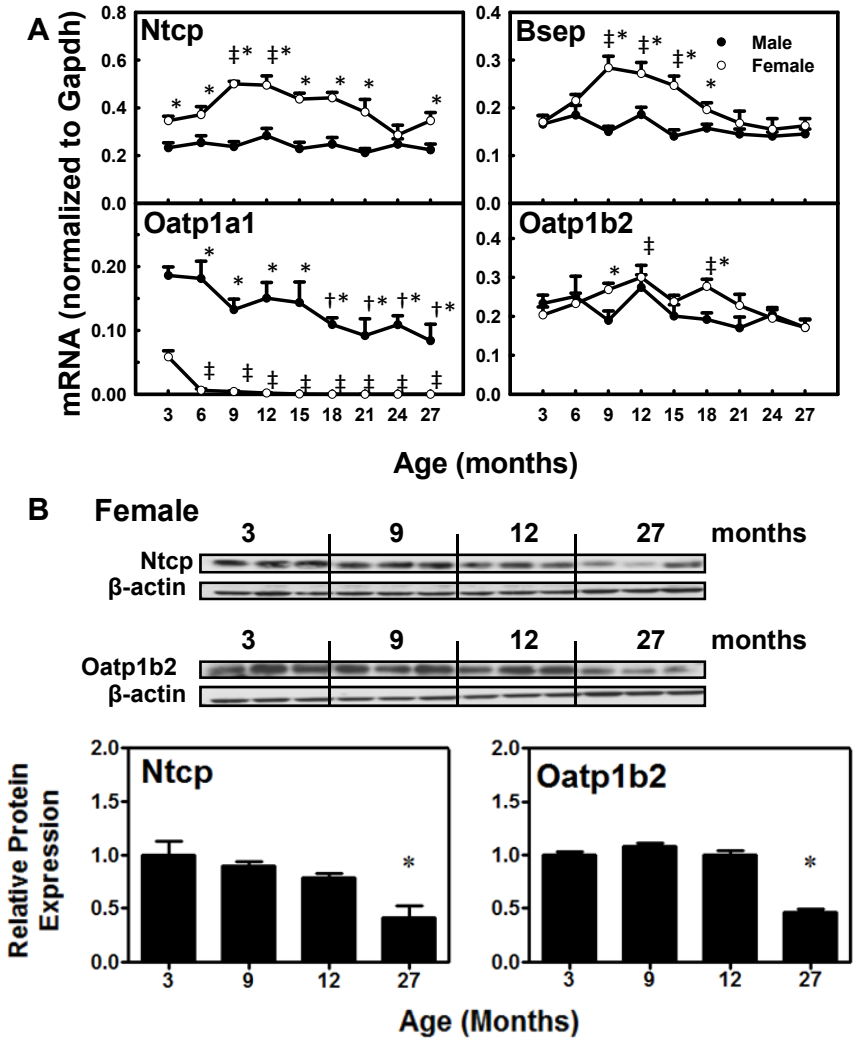
Concentrations of unconjugated BAs in liver during aging of male and female mice. (A) Concentrations of individual unconjugated BAs in liver during aging and (B) proportions of them in all unconjugated BAs in liver at 3 and 27 months of age in male and female mice. Data are presented as means ± SEM of 5-7 mice. Daggers (†) represent statistically significant difference from the value at 3 months of age during aging of male mice. Double daggers (‡) represent significant difference from the value at 3 months of age during aging of female mice. Age-dependent differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. Asterisks (*) represent statistically significant difference between male and female mice at respective ages during aging ($p < 0.05$), by Student's *t*-test. In panel B, pound signs (#) represent differences of BA proportions between 3 and 27 months.

5.3.7 The mRNA and Proteins of BA Hepatic Transporters during Aging.

The mRNA levels of BA uptake transporter Ntcp and efflux transporter Bsep remained relatively constant with age **(Fig. 5.7A)**. Whereas they shared an age-dependent expression pattern in female mice, which was first an increase from 3 to 9 months (Ntcp: 140%; Bsep: 170%) and then a decrease from 9 to 27 months (Ntcp: 30.8%; Bsep: 42.8%). The mRNA level of uptake transporter Oatp1b2 for unconjugated BAs remained constant with age in male mice, whereas in female mice, it increased 150% from 3 to 12 months and decreased 43% thereafter. The mRNA of male-predominantly expressed uptake transporter Oatp1a1 in male mice decreased gradually (54.9%) between 3 and 27 months. Interestingly, Oatp1a1 mRNA in female mice decreased markedly (89.4%) from 3 to 6 months and remained extremely low thereafter.

As shown in **Fig. 5.7B**, decreased proteins in female livers were observed by western blots at 27 months for Ntcp (59%) and Oatp1b2 (53.4%), confirming the decreased expression of these two uptake transporters at a later stage of life in female mice.

Fig. 5.7.



The mRNA and proteins of BA transporters in livers during aging of male and female mice. A. The mRNAs of Ntcp, Bsep, Otap1a1, and Oatp1b2 during aging in male and female livers. Data are presented as means ± SEM of 5-7 mice, by normalization to Gapdh. B. Protein abundance of Ntcp and Oatp1b2 in several representative ages in female livers. Western blots for Ntcp (~50 kDa) and Oatp1b2 (~75 kDa) were performed using liver membrane protein fractions (40 μg protein/lane) from livers of female mice at 3, 9, 12, and 27 months of age. β-Actin (~45 kDa) was used as loading control for each transporter. Bars represent the relative protein expression ± SEM of 3 mice. Daggers (†) represent statistically significant difference from the value at 3 months of age during aging of male mice. Double daggers (‡) represent significant difference from the value at 3 months of age during aging of female mice. Age-dependent differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. Asterisks (*) represent statistically significant difference between male and female mice at respective ages during aging ($p < 0.05$), by Student's *t*-test.

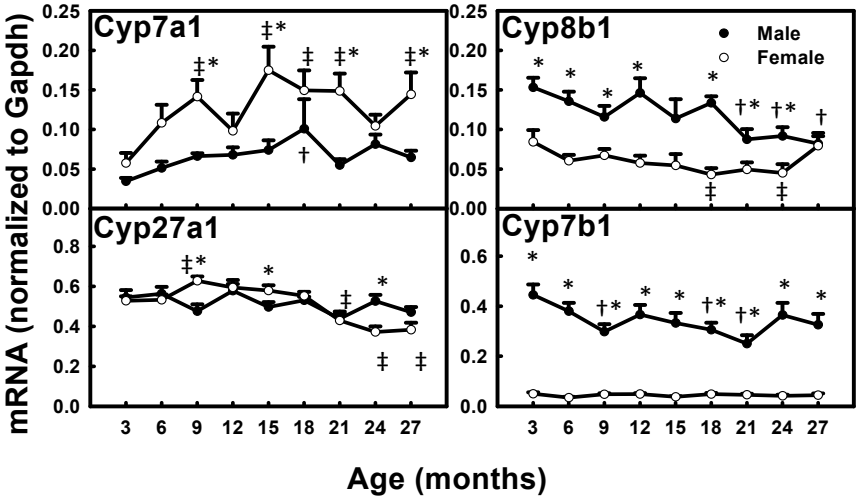
5.3.8 The mRNA changes of BA Synthetic Enzymes in Liver during Aging.

The mRNA of the rate-limiting enzyme Cyp7a1 for BA synthesis increased 290% from 3 to 18 months in male mice and tended to decrease thereafter, whereas in female mice, it increased 300% from 3 to 15 months and remained high thereafter **(Fig. 5.8)**. Cyp8b1 mRNA decreased 46.5% between 3 and 27 months in male mice, whereas in female mice, it decreased 46.6% between 3 and 24 months, and then increased thereafter. Cyp27a1 mRNA level remained relatively constant with age in male mice, whereas in female mice, it increased 120% from 3 to 9 months and then decreased 39.0% thereafter. Cyp7b1 mRNA decreased 43.8% between 3 and 21 months and tended to increase thereafter in male mice, whereas it remained low and constant with age in female mice.

5.3.9 The mRNA changes of Regulators of Cyp7a1 Transcription during Aging.

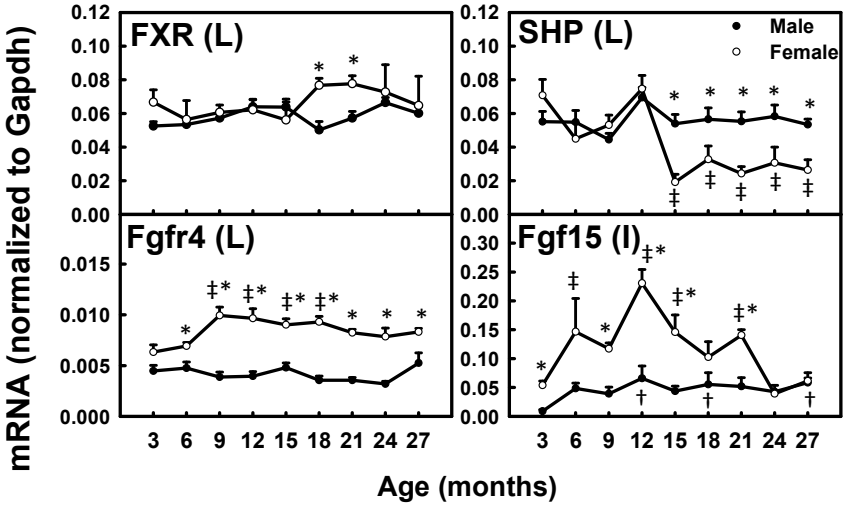
In liver, the mRNA level of FXR remained relatively constant with age in both genders **(Fig. 5.9)**. SHP mRNA level was relatively constant with age in male mice, whereas in female mice, it markedly decreased (74.1%) from 12 to 15 months and remained low thereafter. Fgfr4 mRNA increased (57.1%) from 3 to 9 months and remained high thereafter in female mice, whereas it remained low and constant with age in male mice. In ileum, Fgf15 mRNA gradually increased 670% between 3 and 27 months in male mice, whereas in female mice, it increased 430% between 3 and 12 months and decreased 73.3% thereafter.

Fig. 5.8.



The mRNA changes of BA synthetic enzymes in livers during aging of male and female mice. Data are presented as means ± SEM of 5-7 mice, by normalization to Gapdh. Daggers (†) represent statistically significant difference from the value at 3 months of age during aging of male mice. Double daggers (‡) represent significant difference from the value at 3 months of age during aging of female mice. Age-dependent differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. Asterisks (*) represent statistically significant difference between male and female mice at respective ages during aging ($p < 0.05$), by Student's *t*-test.

Fig. 5.9.



The mRNA changes of regulators of Cyp7a1 transcription during aging of male and female mice. Figure shows mRNA data for FXR, SHP, and Fgfr4 in liver (L) and Fgf15 in ileum (I). Data are presented as means ± SEM of 5-7 mice, by normalization to Gapdh. Daggers (†) represent statistically significant difference from the value at 3 months of age during aging of male mice. Double daggers (‡) represent significant difference from the value at 3 months of age during aging of female mice. Age-dependent differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. Asterisks (*) represent statistically significant difference between male and female mice at respective ages during aging ($p < 0.05$), by Student's *t*-test.

5.4 Discussion

The present study demonstrates a female-specific increase of total BA concentrations in serum during aging of mice. Both conjugated and unconjugated BAs in serum increase with age in female mice, whereas they remain relatively constant with age in male mice **(Fig. 5.1)**. It has been reported that there were no age-related changes between young (8 weeks) and middle-aged (12 months) male rats in biliary BA secretion, distribution of BAs in the bile and intestine, nor the turnover frequency of BAs (Uchida et al., 1990), and in addition, the biliary BA secretion in aged (106 weeks) male rats was similar to younger rats (Uchida et al., 1978). The finding of the present study that concentrations of total BAs in both serum and liver are similar in young and aged male mice is consistent with the previous reports in aged male rats. In humans, fasting concentrations of conjugated and unconjugated serum BAs were similar between 12 younger (mean age 37 years, range 22-59 years; seven female, five male) and 12 elder (mean age 67 years, range 60-82 years; seven female, five male) subjects (Salemans et al., 1993b). This finding in humans is different from the present results in mice, possibly because 1) there are species differences in BA composition and BA conjugation between humans and mice, 2) the small number of subjects compromises the conclusion from the human study, and 3) possible changes of BAs in female subjects may be missed because male and female subjects were not analyzed separately in the human study.

Total BAs in serum and liver become more hydrophilic during aging in both male and female mice. Heuman (Heuman, 1989) and Wang et al. (Wang et al., 2003) have

reported the order of hydrophobicity indices of individual BAs as follows: T-ωMCA (~-0.9) < T-αMCA (-0.84) < T-βMCA (-0.78) < T-MDCA (~-0.6) < T-UDCA (-0.47) < T-HDCA (-0.35) < T-CA (0.00) < T-CDCA (+0.46) <T-DCA (+0.59) < T-LCA (+1.00), ωMCA (~-0.77) < αMCA (~-0.7) < βMCA (~-0.65) < MDCA (~-0.47) < UDCA (-0.31) < HDCA (~-0.22) < CA (+0.13) < CDCA (+0.59) < DCA (+0.72) < LCA (+1.13). The hydrophobicity index (HI) of individual BAs and their proportions in biological samples are used to predict the HI of total BAs at physiological *p*H. Total BAs become more hydrophilic during aging, because the HI of total BAs decreases between 3 and 27 months in serum (M: -0.096 → -0.24; F: -0.095 → -0.20) and liver (M: -0.27 → -0.42; F: -0.32 → -0.38). The decrease is largely due to the increased proportion of hydrophilic βMCA and decreased proportion of hydrophobic DCA **(Fig. 5.2B, Fig. 5.3B, Fig. 5.5B, and Fig. 5.6B)**.

The changes of individual BAs during aging in the present study provide important evidence that BAs may function as markers for longevity. A very intriguing finding in the long-lived lit/lit mice is that they had increased concentrations of several BAs in the serum, and feeding CA to wild-type mice reproduced the expression profiles of xenobiotic metabolism genes observed in the long-lived mice, which might increase resistance to stress and alleviate age-related tissue damage (Amador-Noguez et al., 2007). Females had a longer life expectancy than males in many species, including humans (Vina et al., 2005). Female Wistar rats lived on average 14% longer than males, and female BALB/cJ mice lived much longer than males. The median life span of female mice in a heterogeneous background was shown to be longer than males at

three research sites (Harrison et al., 2009). C57BL/6J mice are long-lived mice compared to other inbred strains, and females tended to live longer than males, but this was not statistically significant, possibly due to the small number of animals (Goodrick, 1975). In the present study, serum concentrations of T-CA, T-αMCA, T-βMCA, T-DCA, and T-UDCA are higher in female C57BL/6 mice, and increase during aging in female mice **(Fig. 5.2A)**. The long-lived lit/lit mice were shown to have increased βMCA, CDCA, LCA, DCA, CA, and UDCA in serum (Amador-Noguez et al., 2007). Therefore, increased serum concentrations of some individual BAs, such as CA, βMCA, DCA, and UDCA might correlate with the tendency of increased longevity in female C57BL/6 mice. UDCA is used clinically to treat gallstones. In cancer cells, UDCA induced senescence through increased histone hypoacetylation and inhibiting telomerase activity (Akare et al., 2006). In the present study, T-UDCA in serum increases 470% between 3 and 27 months of age in female mice **(Fig. 5.2A)**. UDCA in serum increases 800% between 3 and 27 months in male mice, and increases 430% between 3 and 24 months in female mice **(Fig. 5.3A)**. The increased serum concentrations of UDCA during aging indicate that UDCA in serum may be an important marker of longevity.

Interesting, findings from the present study also show gender-divergent changes of expression of BA hepatic uptake and efflux transporters during aging of mice. In female mice, BA uptake transporters Ntcp and Oatp1b2 have decreased expression from about 12 to 27 months **(Fig. 5.7A and B)**, and the BA efflux transporter Bsep has decreased expression from 9 to 27 months of age **(Fig. 5.7A)**. In contrast, in male mice, the expression of Ntcp, Oatp1b2, and Bsep remains constant during aging.

Female-predominant Ntcp expression in young adult mice is due to the inhibitory effect of male-pattern growth hormone secretion (Cheng et al., 2007). The lower sodium-dependent uptake of taurocholate in hepatocytes from female rats was partially due to female-specific lower expression of Ntcp (Simon et al., 1999). The gender-divergent expression changes of Ntcp and Oatp1b2 during aging in the present study might be regulated by gender differences in growth hormone secretion patterns, or changes of female sex hormones with the cease of rodent estrous cycles. In contrast with the changes of BA uptake transporters, the mRNA levels of BA-conjugating enzymes (BAL and BAT) and ileal BA transporters (Asbt and Ostα/β) remain relatively constant with age in both genders (data not shown). Decreased expression of uptake transporters Ntcp and Oatp1b2 likely contributes to the accumulation of BAs in serum with aging in female mice.

In addition to the decreased expression of BA uptake transporters, increased Cyp7a1 expression probably contributes to the increased concentrations of total BAs in serum during aging in female mice. The present study shows Cyp7a1 mRNA increases from 3 to 9 months of age, and remains at a high level thereafter in female mice, whereas in male mice, it remains constant during aging **(Fig. 5.8)**. However, the present data are opposite to previous reports in human livers showing decreased cholesterol 7α-hydroxylation in the elderly (Einarsson et al., 1985; Bertolotti et al., 1993) and an inverse correlation between age and CYP7A1 mRNAs (Bertolotti et al., 2007) in both male and female subjects. There are probably species differences in BA changes during aging between human and mice, and the reliability of the findings in the human

study is compromised by the low correlation coefficient (about -0.6) and the small number of subjects.

The present study characterizes the mRNA changes of several regulators for Cyp7a1 transcription during aging. SHP mRNA level remains at low levels from 15 to 27 months compared to that at 3 months in female mice **(Fig. 5.9)**, and is inversely correlated with Cyp7a1 mRNA during aging in female mice **(Fig. 5.8)**. This can be explained by the down-regulation of Cyp7a1 transcription by the FXR-SHP pathway. The Fgf15-Fgfr4 signaling is also important for down-regulating Cyp7a1 transcription. However, the mRNAs of neither Fgf15 nor Fgfr4 correlate with the elevated Cyp7a1 mRNA **(Fig. 5.9)**. Therefore, in the present models, we conclude that Cyp7a1 transcription appears to be regulated by SHP inhibition during aging.

The current study provides a comprehensive description of the age-related changes of BA composition and concentration in serum and liver of male and female C57BL/6 mice from 3 to 27 months of age. The major findings are that (1) total BA concentrations in serum increase during aging in female mice, whereas they remain relatively constant in male mice; (2) livers maintain constant concentrations of BAs during aging in both genders; (3) total BAs in serum and liver become more hydrophilic during aging in both genders, largely due to the increased proportion of βMCA and decreased proportion of DCA between 3 and 27 months; (4) mRNAs of Ntcp and Oatp1b2 decrease from 9 to 27 months in female mice, whereas they remain relatively constant in male mice; (5) Cyp7a1 mRNA increases from 3 to 9 months and remains at high levels thereafter in female mice, which inversely correlates with SHP mRNA during

aging. In male mice, however, Cyp7a1 mRNA level remains constant with age. Therefore, the female-specific increased total BAs in serum during aging appear to result from a female-specific BA-related gene expression pattern, which is the decreased BA uptake transporters, Ntcp and Oatp1b2, and the increased rate-limiting enzyme for BA synthesis Cyp7a1.

Chapter 6: CALORIE RESTRICTION FEMINIZES THE HEPATIC EXPRESSION OF XENOBIOTIC-METABOLIZING ENZYMES AND TRANSPORTERS IN MICE

6.1 Abstract

CR is one of the most effective anti-aging interventions in various species including mammals. The detoxification theory suggests that aging results from a decline in detoxification capabilities and thus accumulation of damaged macromolecules. Therefore, we hypothesize that hepatic drug metabolism and detoxification are likely altered by CR. Male C57BL/6 mice were fed *ad libitum*, or CR (15, 30, or 40%) diets for one month. Messenger RNA levels of 98 XPGs were quantified in liver, including 7 uptake transporters, 39 phase-I enzymes, 37 phase-II enzymes, 10 efflux transporters, and 5 transcription factors. In general, 15% CR did not alter the mRNA levels of most XPGs, whereas 30 and 40% CR altered over half of the XPGs (32 increased and 29 decreased). CR up-regulated some phase-I enzymes (fold increase), such as Cyp4a14 (12), Por (2.3), Nqo1 (1.4), Fmo2 (5.4), and Fmo3 (346), and numerous phase-II enzymes, such as Sult1a1 (1.2), Sult1d1 (2.0), Sult1e1 (33), Sult3a1 (2.2), Gsta4 (1.3), Gstm2 (1.3), Gstm3 (1.7), and Mgst3 (2.2). CR increased Oatp1a4 mRNA (11), and decreased transporters Oatp1a1 (97%), Mrp3 (42%), and Bcrp (68%). CR feminized the mRNA profiles of 32 XPGs in livers of male mice. The gender-divergent expression of some XPGs is regulated by male-pattern growth hormone, which is decreased by CR. In conclusion, CR alters the mRNA levels of over half of the 98 XPGs quantified in livers of male mice, and over half of these alterations appear to be attributable to feminization of the liver.

6.2 Introduction

CR, defined as reduced calorie intake of a nutritious diet without causing malnutrition, is one of the most effective anti-aging interventions in various species including mammals. Since 1935, when the first study was done in rats (McCay et al., 1935), CR has been known to increase both the mean and maximum lifespan in various species, ranging from yeast, fish, invertebrate animals to hamsters, mice, and dogs. Moreover, CR decreases the incidence of age-related diseases, such as cardiovascular abnormalities and insulin sensitivity in nonhuman primates (Colman et al., 2009) and in humans (Fontana et al., 2004; Redman et al., 2007).

Genome-wide microarray analyses reveal that CR reverses a large proportion of gene expression changes that occur with aging in several tissues, including heart, liver, skeletal muscle, brain, and colon (Han et al., 2000; Lee et al., 2002; Selman et al., 2006). Although several key players (such as insulin/IGF-1, Sirtuins, FoxO, PGC-1α, and autophagy) have been suggested to be important in mediating changes for healthy aging by CR (Martin et al., 2006; Vaquero and Reinberg, 2009; Petrovski and Das, 2010), the mechanism of how CR exerts its anti-aging effects still remain elusive.

The recent detoxification theory of aging suggests that a decline of detoxification capabilities and thus accumulation of damage to macromolecules by reactive intermediates lead to aging (Gems and McElwee, 2005). In alignment with this hypothesis, decreased expression of many genes in xenobiotic metabolism pathways has been reported in livers of aged mice (Cao et al., 2001; Lee et al., 2011; Fu et al., 2012b). Furthermore, microarray studies suggest that xenobiotic metabolism pathways

are one of the gene categories that are altered by CR (Cao et al., 2001; Tsuchiya et al., 2004). Therefore, it was predicted that CR delays aging, at least partially, by up-regulating xenobiotic metabolism and thus increasing xenobiotic resistance (Iqbal et al., 2009).

Our recent study has systematically demonstrated gender differences of XPG expression in livers of mice (Fu et al., 2012b). Gender-divergent gene expression in rodent liver is determined in large part by growth hormone secretory pattern, which is continuous in females and pulsatile in males (Waxman and O'Connor, 2006). Our laboratory has shown that male-pattern growth hormone is an important contributor to the gender-divergent expression of many XPGs, such as Oatps, Sults, Ugts, and Mrps (Cheng et al., 2006; Maher et al., 2006; Buckley and Klaassen, 2009; Alnouti and Klaassen, 2011).

Previous studies on the effect of CR on liver function utilized rats (Apte et al., 2002), long-lived mice models (Tsuchiya et al., 2004), different CR feeding regimens (Selman et al., 2006; Rocha et al., 2007), only provided a global picture by genome-wide microarray (Han et al., 2000; Cao et al., 2001), or focused on some pathway, such as glucose metabolism (Dhahbi et al., 1999; Luo et al., 2008) or anti-oxidant system (De Cabo et al., 2004). The present study was designed to determine the effects of CR on liver xenobiotic processing systems, with respect to the mRNA profiles of major XPGs. The C57BL/6 mouse model, which is used extensively in biomedical research, was chosen for the present study. Utilizing a graded CR model (0, 15, 30, or 40%) where the same housing conditions, same quality of diets, same feeding regimen are guaranteed, reliable

comparison of the effects of graded CR on XPGs is obtained for the first time in the present study, to provide with certainty which xenobiotic metabolizing enzymes and transporters have altered hepatic expression during CR.

6.3 Results

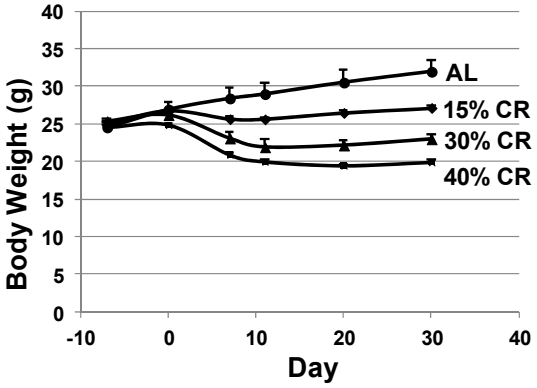
6.3.1 Validation of Graded CR Model.

Previous studies on CR often use one degree of CR (from 15% to 60% CR) and various feeding regimens and diet formulas (Scholmerich et al., 1985; Faulks et al., 2006; Yoshida et al., 2006; Rocha et al., 2007; Boily et al., 2008; Kim et al., 2008b; Niemann et al., 2008; Yamamoto et al., 2009). Therefore, in order to determine which degree of CR altered XPG expression, a graded CR model was utilized in the present dissertation. Duration of one-month of CR was selected because the literature indicates that short-term CR can mimic the majority of gene expression profiles resulting from long-term CR (Cao et al., 2001), and short-term CR (21-day) is sufficient to protect against hepatotoxicity of thioacetamide (Apte et al., 2003).

Effect of Graded CR on Body Weight. As expected, the body weights of CR mice were lower than AL mice, and they reached a "steady state" by the end of the one-month CR feeding **(Fig. 6.1)**. It is very interesting to note that the final body weights of 15%, 30%, and 40% CR mice were 15%, 28%, and 38% lower than AL mice, respectively.

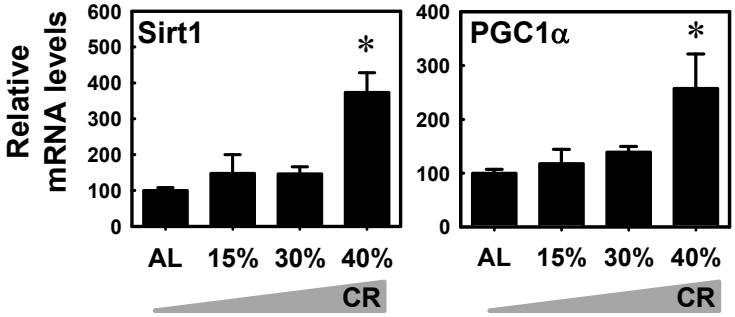
Effect of Graded CR on Hepatic Expression of Typical CR-responsive Target Genes. Sirtuin 1 (Sirt1) and PGC1α are two known CR-responsive target genes. As expected, the mRNAs of Sirt1 (274%) and PGC1a (157%) in liver were up-regulated by CR **(Fig. 6.2)**.

Fig. 6.1.



The body weight changes by graded CR. Body weight was recorded weekly during this study (Day 0 represents the start for one-month CR). The curves show body weight changes in four groups of mice, namely AL, 15%, 30%, and 40% CR. Data are presented as means ± SEM of 5 mice.

Fig. 6.2.



The mRNA changes of CR-responsive target genes in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

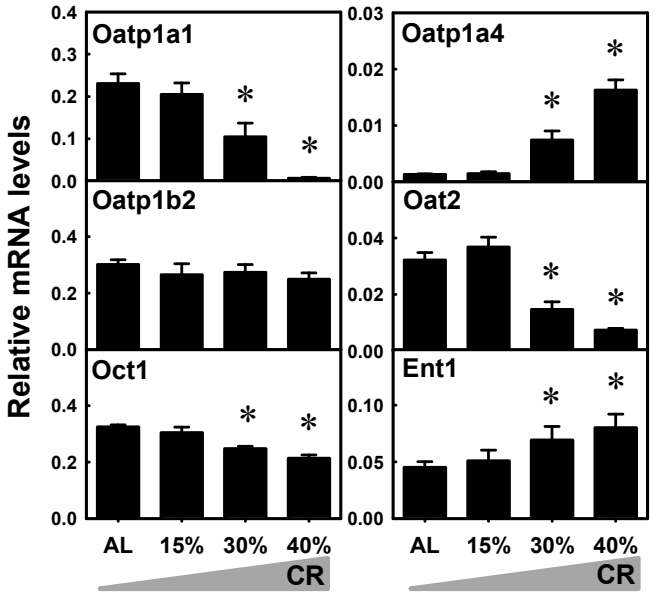
6.3.2 The Effect of Graded CR on Hepatic Expression of Uptake Transporters.

Organic anion-transporting polypeptides (Oatps) are sodium-independent uptake transporters for a wide range of substrates, mainly amphipathic organic compounds, including bile salts, hormones and their conjugates, toxins, and various drugs (Hagenbuch and Gui, 2008). The mRNA of Oatp1a1 was markedly decreased by 30% CR (54.7%) and 40% CR (97.2%) **(Fig. 6.3)**. In contrast, Oatp1a4 mRNA was increased markedly by 30% CR (462%) and 40% CR (11.3-fold). The mRNA level of Oatp1b2 was not altered by CR, as well as Oatp2b1 (data not shown). Organic anion transporter 2 (Oat2) mediates the uptake of various organic anions, such as 5-fluorouracil and paclitaxel (Klaassen and Lu, 2008). Oat2 mRNA was decreased by 30% CR (54.3%) and 40% CR (77.0%). Organic cation transporter 1 (Oct1) plays an important role in the uptake of cationic drugs, such as acetylcholine, acyclovir, cimetidine, and tetraethylammonium (Klaassen and Aleksunes, 2010). Oct1 mRNA was gradually decreased by 30% CR (23.9%) and 40% CR (34.4%). Equilibrative nucleoside transporters (Ents) are sodium-independent, bidirectional facilitated carriers of endogenous nucleosides as well as cancer and antiviral nucleoside analogs (Klaassen and Aleksunes, 2010). Ent1 mRNA was increased by 30% CR (53.4%) and 40% CR (77.8%). In general, CR altered the hepatic expression of 5 of the 7 uptake transporters quantified, increasing two (Oatp1a4 and Ent1) and decreasing three (Oatp1a1, Oat2, and Oct1).

6.3.3 The Effect of Graded CR on Hepatic Expression of Phase-I Enzymes.

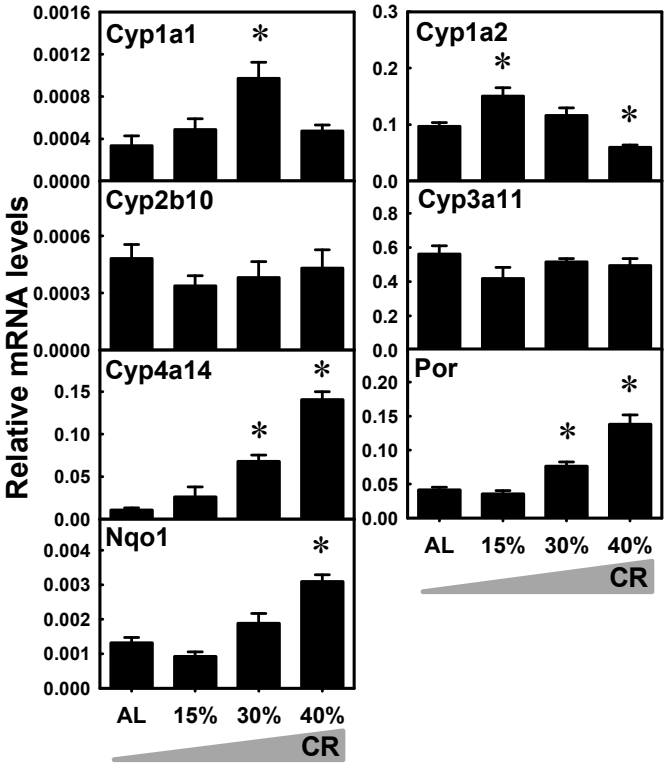
Cytochrome P450s (P450s) are heme-containing monooxygenases. P450s involved in xenobiotic metabolism primarily belong to Cyp1, Cyp2, and Cyp3 families. Substrates for the Cyp4 family are mainly fatty acids and eicosanoids, but also include some xenobiotics (Renaud et al., 2011). The mRNA of Cyp1a1 was increased 191% by 30% CR **(Fig. 6.4)**. Cyp1a2 mRNA was increased 55.3% by 15% CR, but decreased 38.1% by 40% CR. Cyp2b10 and Cyp3a11 mRNA levels were not altered by CR. Cyp4a14 mRNA was increased markedly by 30% CR (534%) and 40% CR (12.1-fold). The mRNA of cytochrome P450 reductase (Por), the electron donor to P450s, was increased by 30% CR (84.1%) and 40% CR (233%). NAD(P)H:quinone oxidoreductase 1 (Nqo1) reduces quinones to hydroquinones, and thus prevents the one electron reduction of quinones that results in free radical production. Nqo1 mRNA was increased 135% by 40% CR. In general, CR altered the hepatic expression of 5 of the 7 P450 related enzymes quantified, increasing Cyp1a1, Cyp4a14, Por, and Nqo1.

Fig. 6.3.



The mRNA changes of uptake transporters in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

Fig. 6.4.



The mRNA changes of P450s, Por, and Nqo1 in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

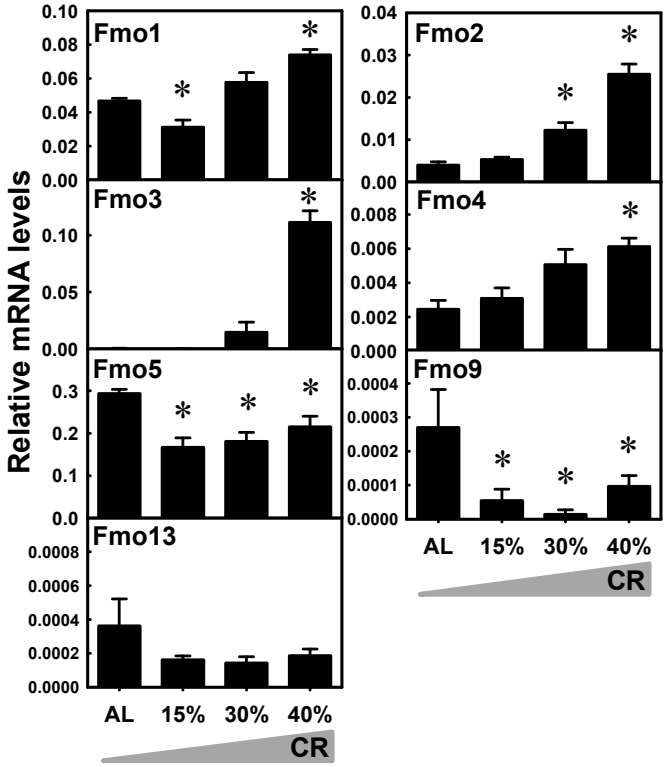
Flavin-containing monooxygenases (Fmos) catalyze the oxidation of endobiotics, as well as a wide range of xenobiotics, including tertiary and secondary alkyl- and arylamines, many hydrazines, thiocarbamides, thioamides, sulfides, disulfides, thiols, and other soft nucleophiles (Hines et al., 1994). Fmo1 mRNA was decreased 33.2% by 15% CR, but increased 58.5% by 40% CR **(Fig. 6.5)**. Fmo2 mRNA was increased by 30% CR (206%) and 40% CR (535%). Fmo3 mRNA was increased 346-fold by 40% CR. Fmo4 mRNA was increased 150% by 40% CR. Fmo5 mRNA was decreased by 15% CR (43.3%), 30% CR (38.5%), and 40% CR (26.8%). Fmo9 mRNA was decreased by 15% CR (79.9%), 30% CR (94.9%), and 40% CR (64.3%). Fmo13 mRNA level was not altered by CR. In general, CR altered the hepatic expression of 6 of the 7 Fmos quantified, increasing 4 (Fmo1, 2, 3, 4) and decreasing 2 (Fmo5 and Fmo9).

Alcohol dehydrogenases (Adhs) and aldehyde dehydrogenases (Aldhs) are pivotal enzymes in metabolizing both endogenous and exogenous alcohols and aldehydes (Cheung et al., 2003). The mRNA of Adh1 was increased slightly by 30% CR (31.6%) and 40% CR (33.2%) **(Fig. 6.6)**. Adh4 mRNA was decreased 36.3% by 40% CR. The mRNA levels of Adh5 and Adh6a were not altered by CR. Adh7 mRNA was decreased 27.9% by 15% CR. Adhfe1 mRNA was decreased 25.9% by 40% CR. As for the aldehyde dehydrogenase family, Aldh3a2 mRNA was decreased by 15% CR (36.4%), 30% CR (33.4%), and 40% CR (33.1%) **(Fig. 6.7)**. Aldh8a1 mRNA was increased by 30% CR (79.9%) and 40% CR (85.7%). The mRNA levels of Aldh1a1, 1a7, 1b1, 2,

4a1, 6a1, 7a1, and 9a1 were not altered by CR. In general, CR had minor effects on the hepatic expression of Adhs and Aldhs.

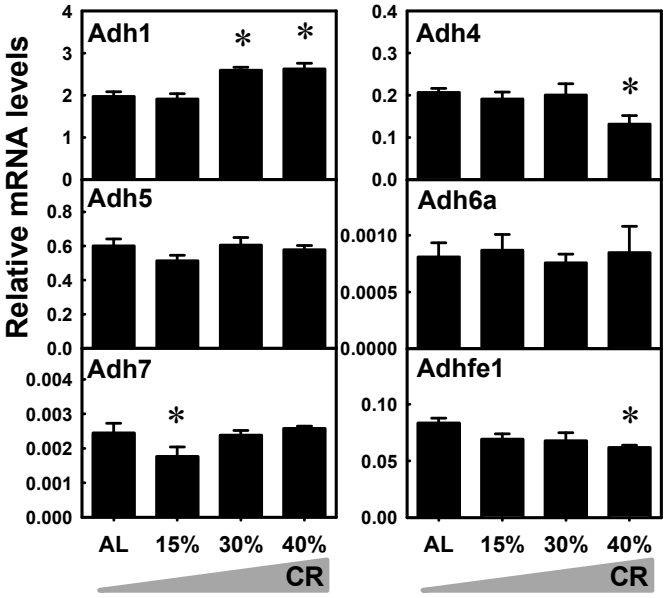
Carboxylesterases (Cess) are important for the hydrolysis of drugs, as well as detoxification of organophosphate and pyrethroid insecticides. Ces1e mRNA was decreased 37.1% by 40% CR **(Fig. 6.8)**. Ces1f mRNA was decreased 46.5% by 40% CR. Ces2c mRNA was decreased markedly by 15% CR (54.3%), 30% CR (83.0%), and 40% CR (91.5%). Ces3a mRNA was decreased by 30% CR (42.3%) and 40% CR (75.5%). The mRNA levels of Ces1c, Ces1g, and Esd were not altered by CR. Paraoxonases (Pons) that catalyze the hydrolysis of organophosphates are also antioxidant enzymes that scavenge oxidized phospholipids (Marsillach et al., 2008). Pon1 mRNA was increased by 30% CR (62.3%) and 40% CR (64.5%). Pon3 mRNA level was not altered by CR. In general, CR altered the hepatic expression of 5 of the 9 Cess and Pons quantified, increasing 1 (Pon1) and decreasing 4 (Ces1e, 1f, 2c, and Ces3a).

Fig. 6.5.



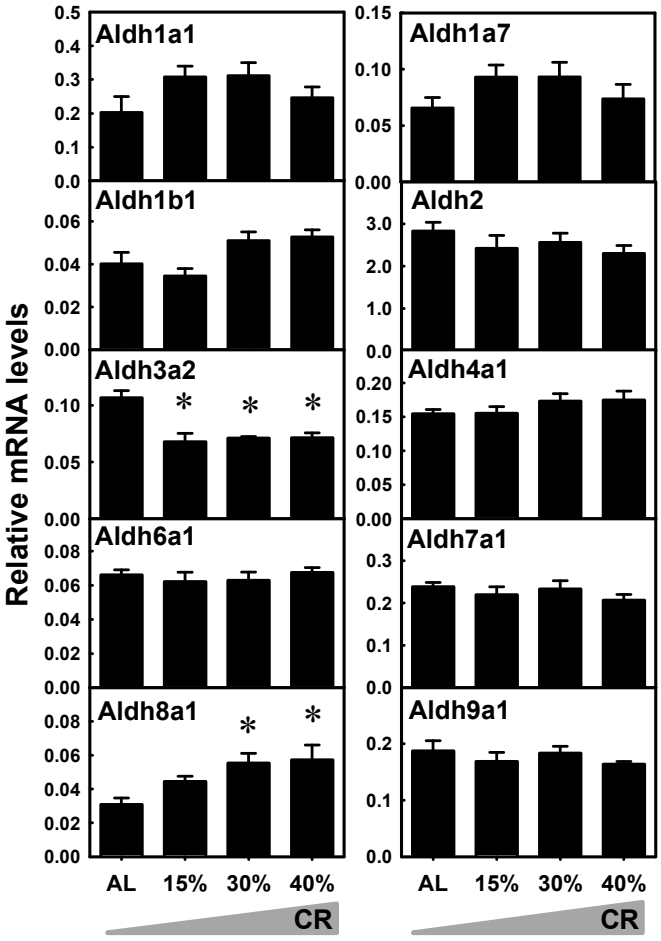
The mRNA changes of Fmos in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

Fig. 6.6.



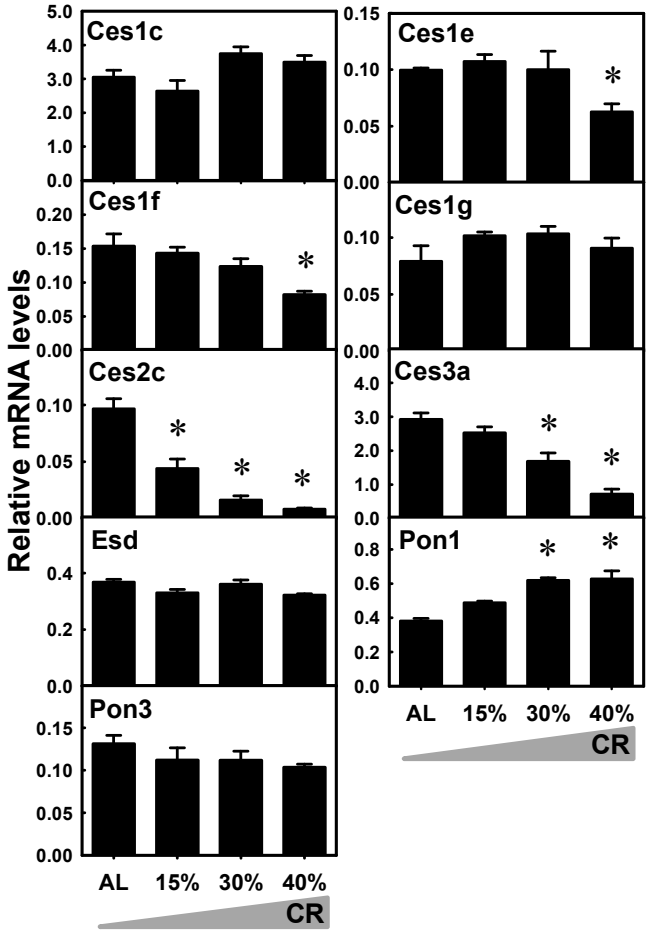
The mRNA changes of Adhs in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

Fig. 6.7.



The mRNA changes of Aldhs in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

Fig. 6.8.



The mRNA changes of Cess and Pons in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

6.3.4 The Effect of Graded CR on Hepatic Expression of Phase-II Enzymes.

Phase−II reactions catalyze conjugation reactions of xenobiotics, most of which enhance the hydrophilicity of xenobiotics, thus favoring their excretion into bile or blood. Sulfotransferases (Sults) catalyze the conjugation of xenobiotics with sulfate. Sult1a1 mRNA was increased by 30% CR (84.6%) and 40% CR (123%) **(Fig. 6.9)**. Sult1d1 mRNA was increased by 30% CR (126%) and 40% CR (204%). Sult1e1 mRNA was increased 33.2-fold by 40% CR. Sult2a1/2 mRNA level was not altered by CR. Sult3a1 mRNA was increased 224% by 40% CR. Sult5a1 mRNA was decreased 57.3% by 40% CR. The sulfate donor for all Sult enzymes is synthesized by two 3'-phosphoadenosine-5'-phosphosulfate synthase (Papss) enzymes. Papss1 mRNA level was not altered by CR. Papss2 mRNA was increased by 30% CR (58.3%) and 40% CR (104%). In general, CR altered the hepatic expression of 6 of the 8 Sults quantified, increasing 5 (Sult1a1, 1d1, 1e1, 3a1, and Papss2) and decreasing 1 (Sult5a1).

UDP-glucuronosyltransferases (Ugts) catalyze the conjugation reaction with glucuronic acid. The mRNA of Ugt1a1 was increased by 30% CR (36.1%) and 40% CR (73.0%) **(Fig. 6.10)**. Ugt1a5 mRNA was increased by 30% CR (80.2%) and 40% CR (76.5%). Ugt1a6a mRNA was decreased by 15% CR (30.3%), 30% CR (23.3%), and 40% CR (30.3%). Ugt2b1 mRNA was decreased by 30% CR (51.0%) and 40% CR (68.5%). Ugt2b36 mRNA was decreased by 15% CR (25.6%), 30% CR (21.2%), and 40% CR (23.8%). Ugt3a1 mRNA was decreased 43.8% by 40% CR. Ugt3a2 was decreased 28.6% by 40% CR. The mRNA levels of Ugt1a9, Ugt2a3, Ugt2b34, and

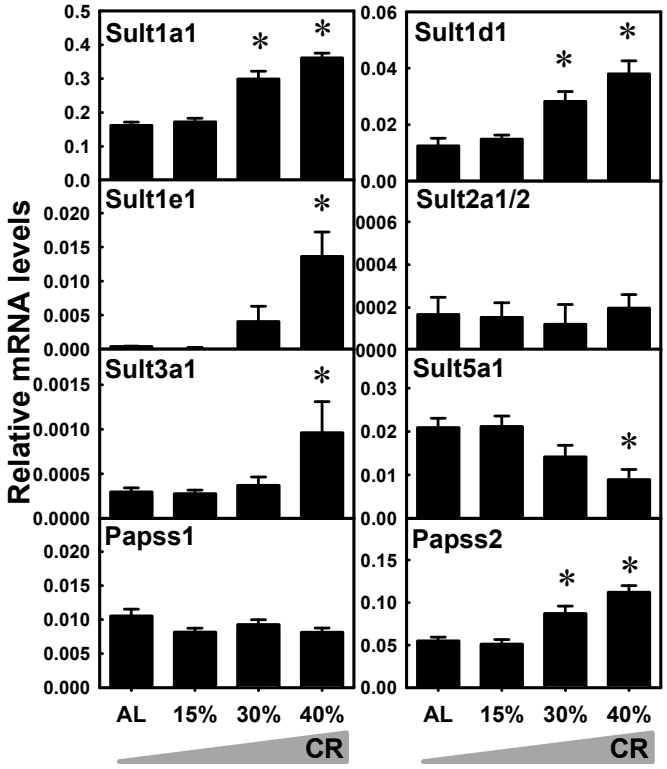
Ugt2b35 were not altered by CR. The co-substrate for glucuronidation, UDP-glucuronic acid, is synthesized by two sequential enzymes, namely UDP-glucose pyrophosphorylase 2 (Ugp2) and UDP-glucose 6-dehydrogenase (Ugdh). Ugp2 mRNA level was not altered by CR (data not shown). Ugdh mRNA was decreased by 15% CR (39.6%), 30% CR (38.7%), and 40% CR (50.1%). In general, CR altered the hepatic expression of 8 of the 13 Ugts quantified, increasing 2 (Ugts1a1, 1a5) and decreasing 6 (Ugt1a6a, 2b1, 2b36, 3a1, 3a2, and Ugdh).

Glutathione-*S*-transferases (Gsts) catalyze the conjugation of chemicals with glutathione. The mRNA of Gsta1 was increased 92.2% by 40% CR **(Fig. 6.11)**. Gsta4 mRNA was increased by 30% CR (50.9%) and 40% CR (132%). Gstm1 mRNA was increased by 30% CR (37.3%) and 40% CR (38.1%). Gstm2 mRNA was increased by 15% CR (49.5%), 30% CR (139%), and 40% CR (131%). Gstm3 mRNA was increased by 30% CR (120%) and 40% CR (174%). Gstm4 mRNA was increased by 30% CR (59.4%) and 40% CR (37.7%). Gstm6 mRNA was increased by 15% CR (48.8%), 30% CR (58.8%), and 40% CR (25.6%). Gstp1 mRNA was decreased markedly by 30% CR (60.1%) and 40% CR (80.8%). Gstp2 mRNA was decreased by 30% CR (37.1%) and 40% CR (50.7%). Gstt1 mRNA was increased slightly by 30% CR (35.6%) and 40% CR (25.7%). Gstt2 mRNA was increased by 30% CR (60.5%) and 40% CR (90.6%). The mRNA of microsomal Gst Mgst1 was decreased by 15% CR (14.5%), 30% CR (25.9%), and 40% CR (41.2%). Mgst3 mRNA was increased by 15% CR (35.3%), 30% CR (151%), and 40% CR (225%). In general, CR altered the hepatic expression of all

13 Gsts quantified, increasing 10 (Gsta1, a4, m1, m2, m3, m4, m6, t1, t2, and Mgst3) and decreasing 3 (Gstp1, p2, and Mgst1).

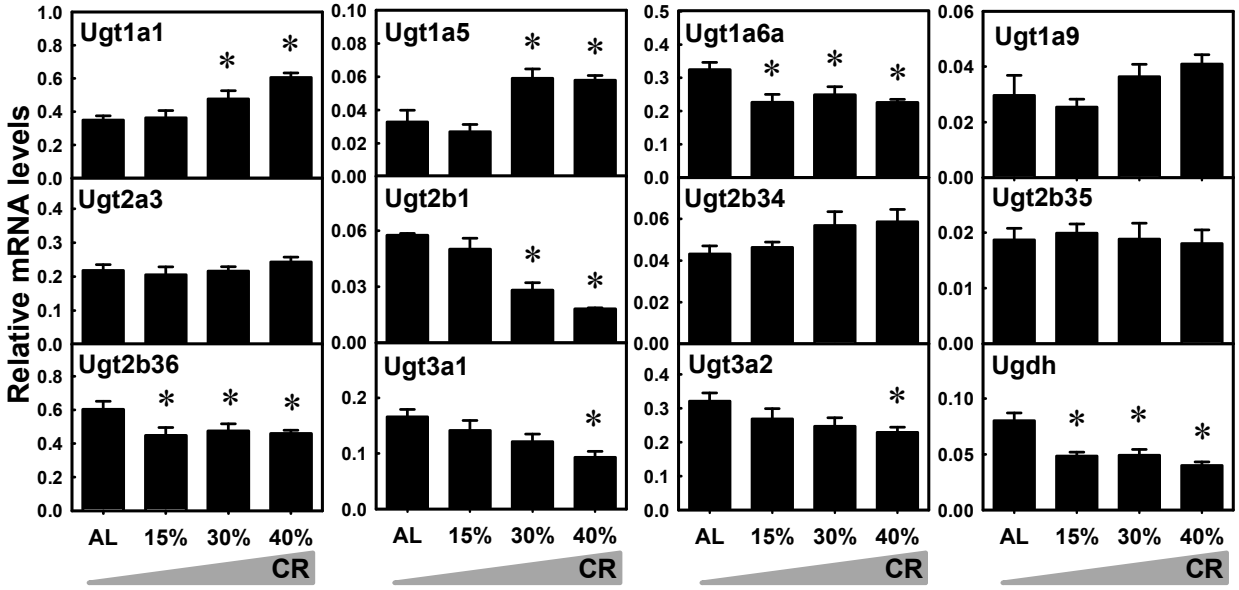
N-acetyltransferases (Nats) and catechol-*O*-methyltransferase (Comt) catalyze conjugation reactions with acetyl and methyl groups, respectively. In contrast to the other conjugation pathways, these two pathways decrease the hydrophobicity of the substrates. The mRNA levels of Nat1 and Nat2 were not altered by CR **(Fig. 6.12)**. The mRNA of Comt was decreased by 30% CR (52.2%) and 40% CR (64.6%). In general, CR decreased the hepatic expression of Comt but did not alter the expression of Nats.

Fig. 6.9.



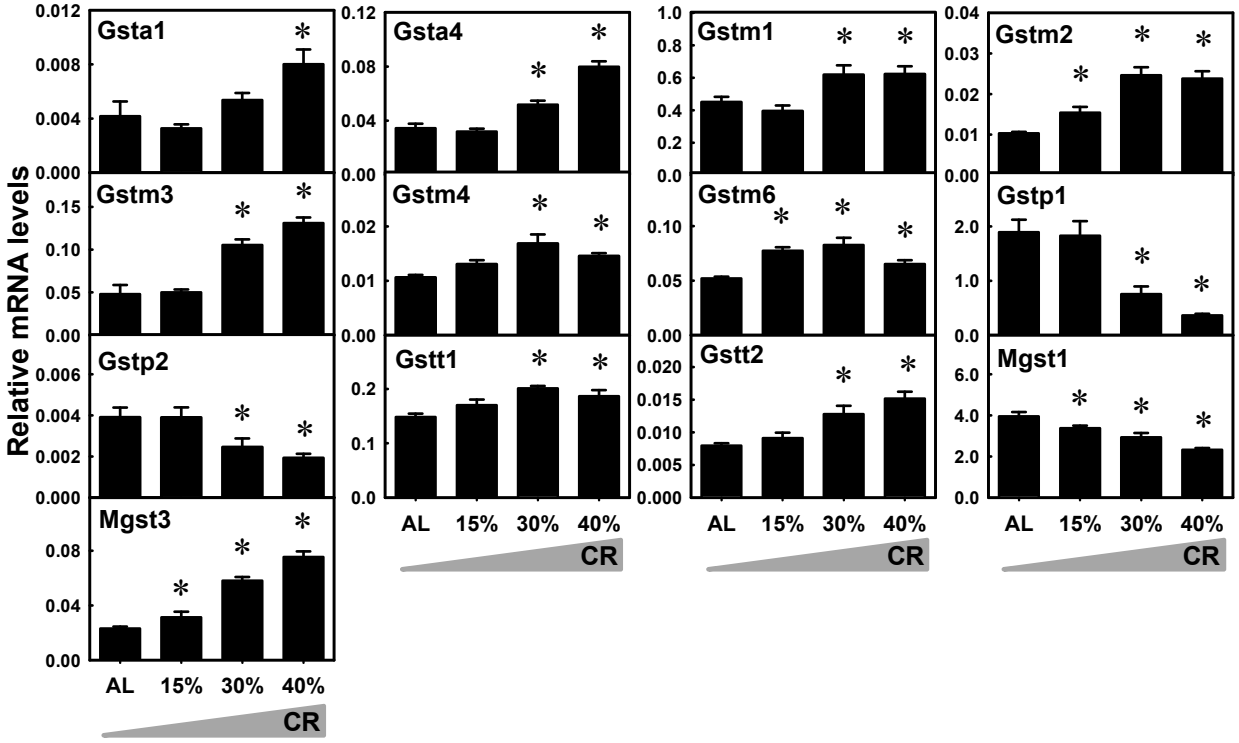
The mRNA changes of Sults and Papss in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

Fig. 6.10.



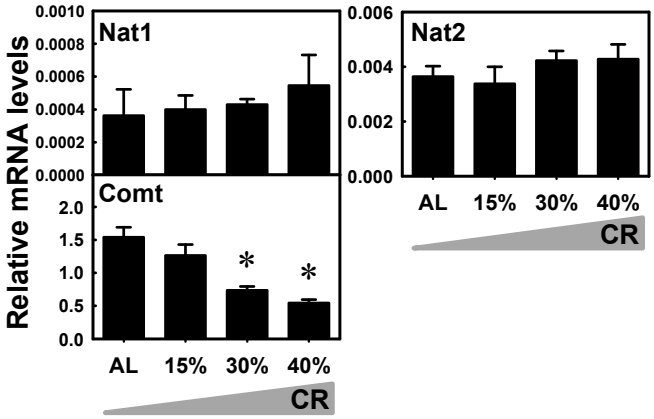
The mRNA changes of Ugts and Ugdh in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

Fig. 6.11.



The mRNA changes of Gsts in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

Fig. 6.12.

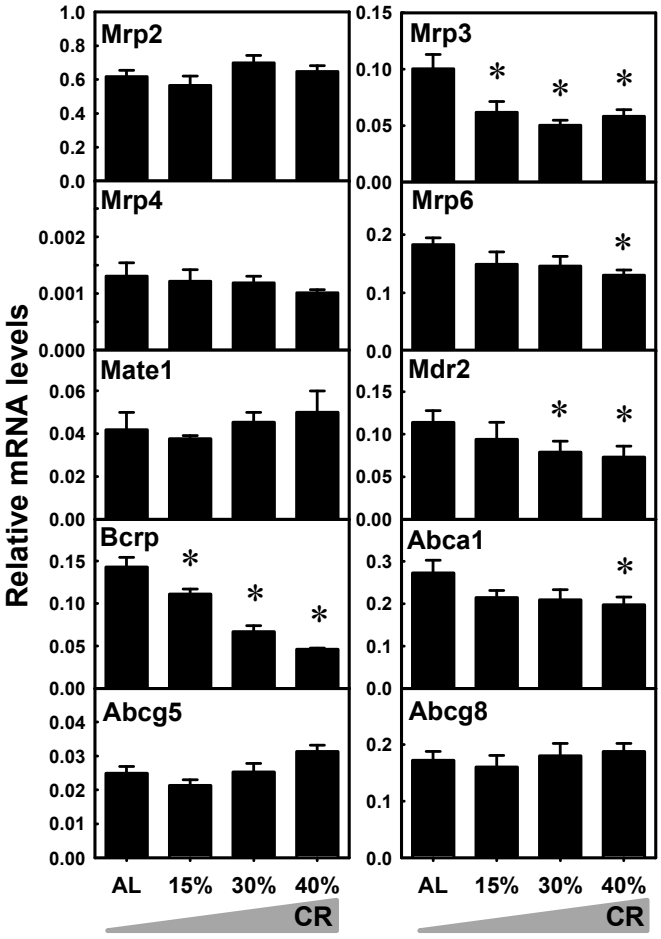


The mRNA changes of Nats and Comt in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

6.3.5 The Effect of Graded CR on Hepatic Expression of Efflux Transporters.

Efflux transporters play a key role in the elimination of xenobiotics. Multidrug resistance-associated protein 3 (Mrp3) is important in retro-transporting bilirubin glucuronides and bile acids back into blood, whereas breast cancer resistant protein (Bcrp) is important for transporting sulfate and glucuronide conjugates into bile. The mRNA of Mrp3 was decreased by 15% CR (38.3%), 30% CR (49.7%), and 40% CR (41.8%) **(Fig. 6.13)**. Mrp6 mRNA was decreased 28.5% by 40% CR. Multidrug resistance protein 2 (Mdr2) mRNA was decreased by 30% CR (30.8%) and 40% CR (36.0%). Bcrp mRNA was decreased gradually by 15% CR (22.4%), 30% CR (53.3%), and 40% CR (67.8%). Abca1 mRNA was decreased 27.7% by 40% CR. The mRNA levels of Mrp2, Mrp4, Mate1, Abcg5, and Abcg8 were not altered by CR. In general, CR altered the hepatic expression of 5 of the 10 efflux transporters quantified, decreasing Mrp3, 6, Mdr2, Bcrp, and Abca1.

Fig. 6.13.

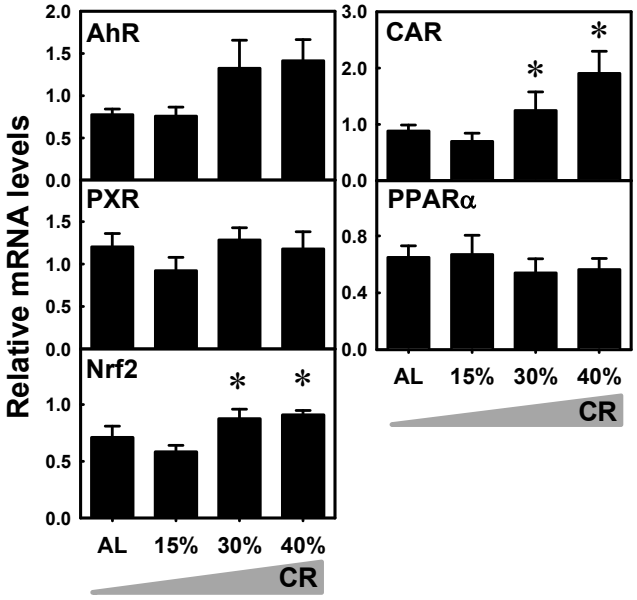


The mRNA changes of efflux transporters in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

6.3.6 The Effect of Graded CR on Hepatic Expression of Transcription Factors.

The expression of many XPGs are regulated by transcription factors, such as the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor alpha (PPARα), and nuclear factor E2-related factor 2 (Nrf2) (Klaassen and Slitt, 2005). These transcription factors are responsive to xenobiotics and their activation leads to increased or decreased expression of various XPGs (Klaassen and Aleksunes, 2010). In order to determine the regulation of XPGs in this graded CR model, mRNAs of major transcription factors were quantified in livers. The mRNA of AhR tended to be increased by 30% and 40% CR, but was not statistically significant **(Fig. 6.14)**. The mRNA of CAR was increased by 30% CR (41.2%) and 40% CR (116%). The mRNA of Nrf2 was increased by 30% CR (23.2%) and 40% CR (28.1%). The mRNA levels of PXR and PPARα were not altered by CR. In general, among the transcription factors quantified, CAR and Nrf2 were the only two whose hepatic expression was increased by CR.

Fig. 6.14.



The mRNA changes of several transcription factors in livers by graded CR. Data are presented as means ± SEM of 5 mice. Asterisks (*) represent statistically significant differences were considered at $p < 0.05$ by one-way ANOVA, followed by Duncan's post-hoc test. The triangle marks represents increased calorie restriction (CR).

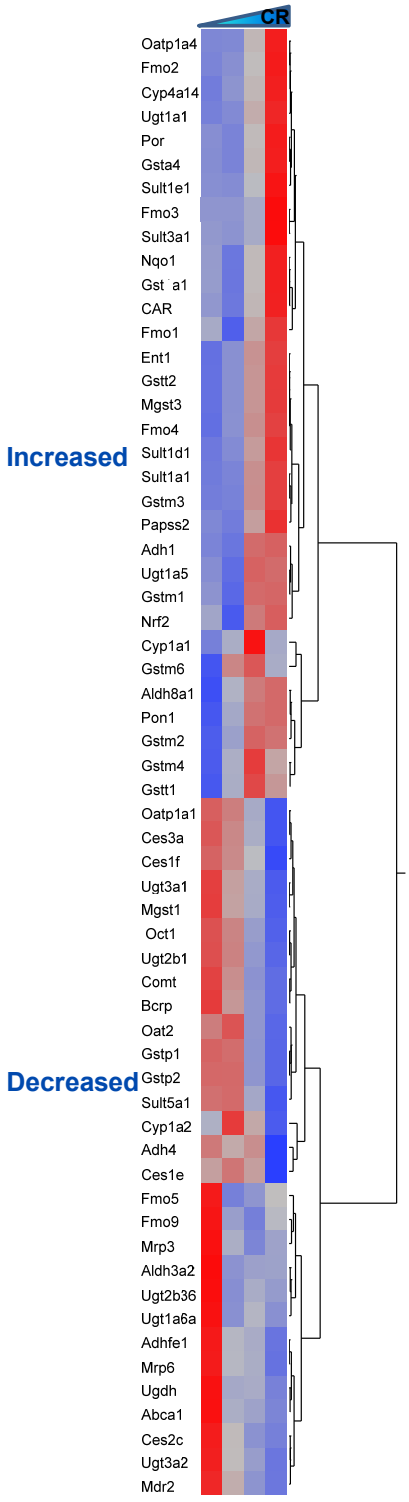
6.4 Discussion

The present study demonstrates that 15% CR is not sufficient to alter the mRNA levels of most XPGs, whereas 30 and 40% CR alter over half of the XPGs (61 of 98), with 32 being increased and 29 decreased **(Fig. 6.15)**. XPGs that are markedly altered by CR belong to the Oatp, Fmo, Sult, and Gst families.

In general, uptake transporters Oatp1a1 and Oatp1a4 demonstrate overlapping substrate specificity *in vitro*, such as sulphobromopthalein, sulfated steroids, β-lactam antibiotics, and fexofenadine. However, Oatp1a4 has unique substrates such as digoxin (Klaassen and Aleksunes, 2010). Interestingly, these two Oatps are oppositely regulated by CR **(Fig. 6.3)**. Further studies are needed to conclude the effects of CR on the transport of various Oatp substrates.

Several P450s are up-regulated by CR. Noticeably, Cyp4a14 mRNA is increased 12 fold in the present study **(Fig. 6.4)**, which is greater than the increase (2-fold) in long-lived C3B10RF1 mice with long-term CR (Cao et al., 2001), but smaller than the increase (50-fold) in 129/SV mice after 24 h starvation (Bauer et al., 2004). Various mice strains and dietary regimens probably contribute to these discrepancies. The up-regulation of Cyp4a14, an omega hydroxylase of arachidonic acids (Okita and Okita, 2001), reflects the increased requirement for fatty acid oxidation in liver to generate energy.

Fig. 6.15.



Continued to the following page.

Fig. 6.15. (continued)

Hierarchical clustering of the mRNA profiles of XPGs in livers by graded CR. Clustering analysis results are shown in the dendrogram, with y-axis representing the XPGs that change with graded CR ($p < 0.05$, one-way ANOVA) and x-axis representing the graded CR (0, 15, 30, and 40%). In the heatmap, the red color represents relatively high mRNAs and blue represents relatively low mRNAs. The spectrum of each gene is standardized and specific to the scale of its own mRNA. Therefore, it's not valid to compare the mRNA levels among different genes according to the color.

In addition to P450s, Fmos are another phase-I enzyme family whose expression is altered markedly by CR. Fmos typically make xenobiotic substrates more polar, less pharmacologically active, and more easily excreted (Cashman, 2000). Human FMO3 contributes to the metabolic clearance of a variety of drugs, such as cimetidine, nicotine, and tamoxifen, as well as the diet-derived substrate trimethylamine (Cashman, 2000). The current finding that CR markedly up-regulates Fmo3 (346-fold; **Fig. 6.5**) may suggest a marked increase in oxidative metabolism and clearance of its substrates.

CR up-regulates numerous phase-II enzymes, including many Sults, some Ugts, and most Gsts. Phase-II conjugation by these three enzyme families increases the hydrophilicity of xenobiotics and promotes eventual excretion of their polar metabolites, thus playing crucial roles in detoxification. Sult1e1 sulfonates a variety of estrogens, and is the predominant determinant of the ratio of free estrogens to inactive sulfate conjugates. The inhibition of CR on mammary tumorigenesis is thought to be mediated in part by suppression of estrogen levels (Sylvester et al., 1982). This correlates with the marked elevation of Sult1e1 (33.2-fold) by CR **(Fig. 6.9**, and thus possibly increased estrogen sulfation. Modest up-regulation of Sult1a1 and Sult1d1 by CR **(Fig. 6.9)** suggests a possible increase in sulfation and clearance of phenolic substrates (such as acetaminophen and troglitazone) (Runge-Morris et al., 2013). Sult3a1 is the only Sult that catalyzes *N*-sulfonation, rather than *O*-sulfonation of amines, such as the neurotoxicant phenyltetrahydropyridine and the rodent carcinogen 4-chloroaniline (Yoshinari et al., 1998). The modest increase of Sult3a1 by CR **(Fig. 6.9)** suggests possible elevation of metabolism and detoxification of these toxic substrates.

In comparison to Sults, Ugts are altered by CR to a lesser degree. Glucuronosyltransferases in liver are important for deactivating the biological activities of thyroid hormones (Barter and Klaassen, 1994), which are the master regulator of basal metabolic rate. CR-induced increase of Ugt1a1 **(Fig. 6.10)**, a glucuronosyltransferase for the conjugation of thyroid hormones (Findlay et al., 2000), correlates with a previous report that CR decreases thyroid hormones (Fontana et al., 2006). Increased Ugt1a1 and Ugt1a5 by CR in the present study are consistent with a previous finding that mutants of *C. elegens* with spontaneous CR have increased expression of Ugts, to potentially dispose of toxic endobiotic and xenobiotic compounds to ensure longevity (McElwee et al., 2004). Therefore, elevated detoxification might be an evolutionarily conserved anti-aging pathway. In contrast to modest increases in Ugt1a1 and Ugt1a5, CR down-regulates several Ugts, including Ugt1a6a, 2b1, 2b36, 3a1, 3a2, and Ugdh **(Fig. 6.10)**. Further studies are needed to investigate the significance of the down-regulation of these Ugts by CR.

Up-regulation of Gsts appears to be a common pathway associated with longevity. Gsts conjugate a wide range of electrophilic compounds, promote their excretion, and thus detoxify electrophiles by preventing the damage to macromolecules. Therefore, Gsts have been proposed as a candidate to assure longevity in multiple species (McElwee et al., 2007). A comparison of the mRNA profiles of XPGs in livers of genetic long-lived models, namely Little mice and Ames dwarf mice (Amador-Noguez et al., 2004), as well as dietary long-lived model CR mice (data from the present study), shows that common changes include increased Oatp1a4, Cyp4a14, Fmo3, Sult1a1, Papss2,

Gsta4, m3, t1, and Mgst3, and decreased Oatp1a1, Ces3a, Gstp2, and Comt. Noticeably, many of these XPGs belong to the Gst family. Gsta4 plays crucial roles in detoxifying 4-hydroxy-2-nonenal, which is a major lipid peroxidation product known to cause oxidative stress and to accumulate during aging (Sohal and Weindruch, 1996). The increased expression of Gsta4 (about 2-fold) by CR **(Fig. 6.11)** may provide a potential anti-aging mechanism of CR, which is supported by the evidence that overexpression of mouse Gsta4 in *C. elegans* increases stress resistance and life span (Ayyadevara et al., 2005). Among the various Gsts, the Pi class has attracted attention because high levels of Gsts have been linked with cancer incidence (Tew and Townsend, 2011). In contrast to the general up-regulation of Gsts, Gstp1 and Gstp2 are down-regulated by CR **(Fig. 6.11)**, which correlates with lower cancer incidence in CR rodents and monkeys (Longo and Fontana, 2010).

Transcription factors may not play leading roles in the regulation of CR-induced changes of XPGs. AhR, PXR, and CAR do not appear to be activated by CR, because the expression of their prototypical target genes (Cyp1a2, Cyp3a11, and Cyp2b10, respectively) (Klaassen and Aleksunes, 2010) are not induced **(Fig. 6.4)**. Although CAR has been suggested to mediate the up-regulation of some phase-II enzymes by fasting (Maglich et al., 2004), our study and a previous report (Cao et al., 2001) exclude the possibility of CAR activation by CR in liver, due to the lack of increases in mRNAs of CAR target genes Cyp2b10 and Sult2a1. PPARα, a regulator for fatty acid oxidation, may be activated by CR in liver, because of the increase in its prototypical target gene Cyp4a14 **(Fig. 6.4)**, however, the role of PPARα in the regulation of drug metabolism is

minor. Nrf2, a master regulator for antioxidant defense and electrophile detoxification, might be activated by CR in liver. Because some Nrf2 target genes (such as Nqo1 and many Gsts) (Klaassen and Aleksunes, 2010; Wu et al., 2012) are up-regulated by CR **(Fig. 6.4 and Fig. 6.11)**, in addition to the increase of Nrf2 mRNA **(Fig. 6.14)**. However, another Nrf2 target gene Mrp3 is down-regulated by CR **(Fig. 6.13)**. In summary, the CR-induced changes of XPGs do not appear to be regulated by a single transcription factor.

Previous studies have shown that CR can feminize gene expression profiles in male mice (Estep et al., 2009) and long-lived mice have lost the sex dimorphism of gene expression (Amador-Noguez et al., 2005). Comparing with our previous report on gender differences of XPG expression in livers of mice (Fu et al., 2012a), the present study indicates that CR feminizes the mRNA profiles of 32 XPGs, which is over half of the XPGs that are altered by CR. For example, male mice have higher expression of Oatp1a1 in liver (Cheng et al., 2005), and Oatp1a1 mRNA is decreased markedly by 40% CR (97.2%) **(Fig. 6.3)**. In contrast, male mice have lower expression of Oatp1a4 in liver (Cheng et al., 2005), and Oatp1a4 mRNA is markedly increased by 40% CR (11.3-fold) **(Fig. 6.3)**. Both CR-induced decrease in male-predominant Oatp1a1 and increase in female-predominant Oatp1a4 are attributable to feminization effects by CR in male mice. Feminization is also observed for male-predominantly expressed Ces2c, 3a, Ugt1a6a, 2b1, Gstp1, p2, and Bcrp, as well as female-predominantly expressed Ent1, Cyp1a1, 4a14, Por, Nqo1, Fmo1-4, Aldh8a1, Pon1, Sult1a1, 1d1, 3a1, Papss2, Ugt1a1, 1a5, Gsta4, m2, t1, Mgst3, CAR, and Nrf2 **(Table 6.1)**. However, the

remainder of the XPGs that are altered by CR either do not have gender-divergent expression (19 XPGs) or their changes by CR fail to be explained by feminization (10 XPGs).

Pulsatile growth hormone secretory pattern in males mediates gender differences of many XPGs. CR was reported to decrease plasma growth hormone levels in males (Chacon et al., 2005). Therefore, the feminization effects of many XPGs by CR in males are likely attributable to the CR-induced decrease in growth hormone levels. In general, humans have much smaller gender differences in XPG expression than rodents (Waxman and O'Connor, 2006). Thus, one needs to be cautious when extrapolating the significance of feminization of XPG expression in liver by CR from mice to humans.

In conclusion, the present study used a graded CR model and compared the mRNA profile changes of 98 XPGs in livers of male mice by CR. The major findings are 1) CR (30 and 40%) altered over half of the XPGs; 2) CR up-regulated 17 phase-II drug metabolizing enzymes; and 3) over half of the XPGs that are altered by CR appear to be due to feminization of the liver by CR. Results from the present study on the alteration of XPGs by CR sheds light on the potential mechanisms of the anti-aging effects of CR, and may indicate which XPG pathways are altered, thus the dosing, pharmacokinetics, and toxicity of drugs might be altered in people on diets.

Table 6.1. CR (30 and 40%) feminizes the mRNA profiles of over half of the XPGs that are altered in livers of male mice.

	mRNA changes by CR	List of XPGs (Category: Name)	♂ or ♀ predominant expression
"Feminization effects"	↓	Uptake transporter: Oatp1a1	♂
		Phase-I enzymes: Ces2c, Ces3a	
		Phase-II enzymes: Ugt1a6a, Ugt2b1, Gstp1, Gstp2	
		Efflux transporter: Bcrp	
	↑	Uptake transporter: Oatp1a4, Ent1	♀
		Phase-I enzymes: Cyp1a1, Cyp4a14, Por1, Nqo1, Fmo1, Fmo2, Fmo3, Fmo4, Aldh8a1, Pon1	
		Phase-II enzymes: Sult1a1, Sult1d1, Sult3a1, Papss2, Ugt1a1, Ugt1a5, Gsta4, Gstm2, Gstt1, Mgst3	
		Transcription factors: CAR, Nrf2	

♂: male
♀: female

Chapter 7: REGULATION OF BILE ACID ENTEROHEPATIC CIRCULATION BY GRADED CALORIE RESTRICTION IN MICE

7.1 Abstract

Recent reports have suggested a connection between longevity and BAs, which are metabolites of cholesterol. However, how BA metabolism is regulated by a well-known anti-aging intervention CR is not known. Therefore, the present study quantified 20 individual BAs in various compartments of the enterohepatic circulation by UPLC-MS/MS in a "dose-response" model of CR (0, 15, 30, or 40% CR for one month, C57BL/6 male mice). CR (40%) increased the BA pool size (162%) and total BA concentrations in serum, gallbladder, and small intestinal contents. Graded CR "dose-dependently" increased the concentrations of tauro-cholic acid (TCA) and many secondary BAs in serum, such as TDCA, DCA, lithocholic acid, ω-muricholic acid (ωMCA), and hyodeoxycholic acid. Notably, 40% CR increased tauro-deoxycholic acid (TDCA) concentrations over 10 fold (serum, liver, and gallbladder). CR (40%) increased the proportion of 12-hydroxylated BAs (CA and DCA), which correlated with the improved glucose tolerance and lipid parameters. The increase in BAs correlated with increased expression of BA-synthetic (Cyp7a1) and conjugating enzymes (BAL), and the ileal BA-binding protein by 40% CR. These results demonstrate that CR increases BAs possibly through orchestrated increases in BA synthesis by the classical pathway, as well as BA conjugation in liver and intracellular transport in ileum.

7.2 Introduction

CR is the most effective intervention to increase longevity in mammals. CR increases the lifespan of various species ranging from yeast, fish, invertebrate animals to hamsters, mice, rats, and dogs (Masoro, 2005). Moreover, CR decreases the incidence of aging-related diseases in nonhuman primates (Colman et al., 2009) and human beings (Fontana et al., 2004; Johannsen et al., 2007). However, the exact mechanism underlying the anti-aging effects of CR remains elusive.

BAs are important endogenous molecules in the enterohepatic system. Synthesized from cholesterol in the liver, BAs regulate cholesterol homeostasis, and facilitate intestinal absorption of dietary lipids (Hofmann and Hagey, 2008). Recently, BAs have been shown to be signaling molecules, regulating the homeostasis of BAs (Song et al., 2011), lipids (Sinal et al., 2000; Sirvent et al., 2004), glucose (Scholmerich et al., 1985; Staels and Kuipers, 2007), and energy (Watanabe et al., 2006; Li et al., 2012). FXR is closely involved in the metabolism of lipids (Sinal et al., 2000; Sirvent et al., 2004) and glucose (Scholmerich et al., 1985; Staels and Kuipers, 2007). On its activation by BAs, FXR regulates bile acid synthesis, conjugation, and transport, as well as glucose and lipid metabolism (Claudel et al., 2005). In addition, BAs are ligands for the G-protein-coupled receptor TGR5 (Maruyama et al., 2002), through which BAs can increase energy expenditure in brown adipose tissue and muscle (Watanabe et al., 2006).

Connection between longevity and bile acids (BAs) has been indicated by some recent reports. In long-lived lit/lit mice, higher levels of BAs activated xenobiotic

metabolizing gene expression possibly via FXR (Amador-Noguez et al., 2007), and the authors suggested that this increased resistance to xenobiotic stress possibly contributes to the longevity. This finding indicates that BAs may be a longevity signal in long-lived lit/lit mice (Gems, 2007). In addition, a chemical screen identified LCA as an anti-aging molecule that extended the life span of yeast (Goldberg et al., 2010b). Moreover, 3-keto BA-like steroids, called dafachronic acids, increase the life span of *C. elegans*, through DAF-12 (a homolog of FXR) (Held et al, 2006; Gerisch et al., 2007). Our laboratory also recently reported that female mice have higher BA concentrations in serum than males during aging (Fu et al., 2012b), which correlates with the tendency that female mice live longer. These studies suggest a link between BAs and longevity.

Because CR is the most effective anti-aging intervention in mammals, we hypothesized that BA concentrations would be higher in CR mice. Therefore, the present study was designed to determine the effect of CR on BA homeostasis by varying the extent of CR (0, 15, 30, or 40%), and BA quantification in various compartments of the EHC by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Furthermore, the mRNAs of BA-related genes were quantified to reveal possible mechanisms for BA alterations by CR.

7.3 Results

7.3.1 Body Weight, Glucose, and Lipid Parameters in CR Mice.

In order to validate the graded CR model, body weight was recorded weekly. As expected, the body weights of CR mice were lower than AL mice, and they reached a "steady state" by the end of the study **(Fig. 7.1A)**. Interestingly, the final body weights of 15%, 30%, and 40% CR mice were all much lower than AL mice, which was 15%, 28%, and 38%, respectively. The health condition of CR mice were monitored throughout the study, and no malnutrition was apparent. As shown in **Fig. 7.1B**, CR mice had better insulin sensitivity, evidenced by lower AUCs in glucose tolerance test (GTT). In addition, 30 and 40% CR mice had lower blood glucose than AL mice before glucose challenge as well, but they were not hypoglycemic. Lipid parameters in serum and liver of AL and CR mice were examined. TG levels in plasma were decreased by 40% CR, and interestingly, the levels in liver were decreased by CR proportionally. Moreover, cholesterol and FA levels were decreased by CR in plasma and liver **(Fig. 7.1C)**.

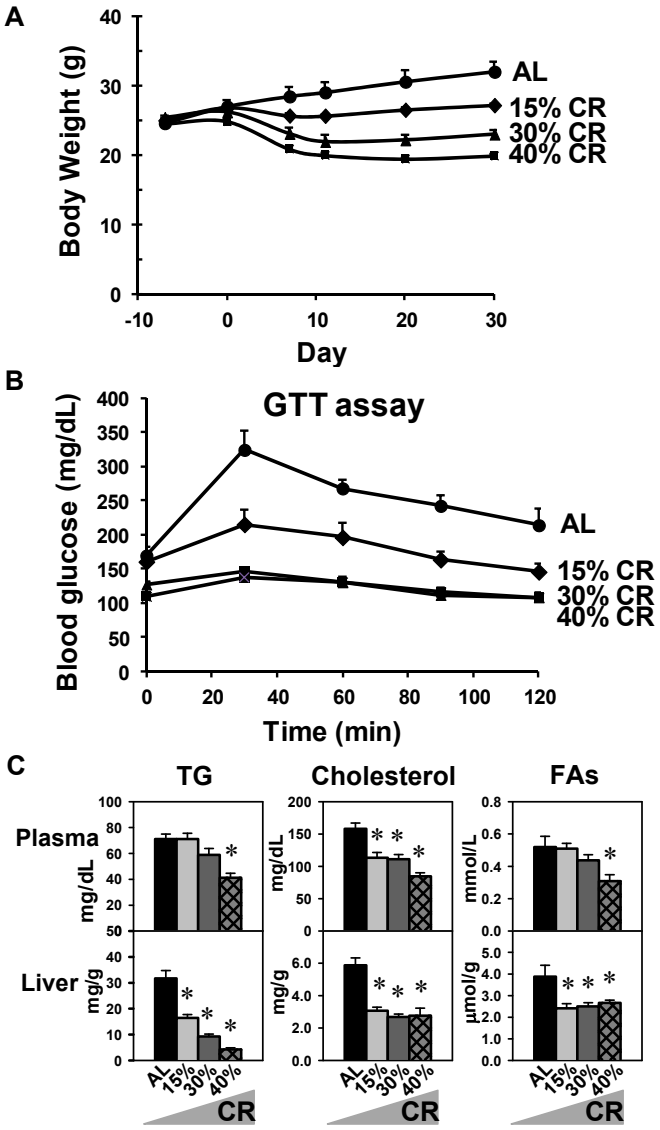
7.3.2 Effect of Graded CR on Total BAs in Various Compartments of the Enterohepatic Circulation.

Serum, liver, gallbladder (GB), small intestine (SI), and large intestine (LI) are the major compartments of the enterohepatic circulation (EHC) of BAs. BA pool size is estimated by adding the BA amounts in liver, GB, and intestine. Forty percent CR increased BA pool size (162%), due to the increased BA amounts in GB (133%) and SI

(174%) **(Fig. 7.2, top-left panel)**. However, CR did not alter BA amounts in liver and LI.

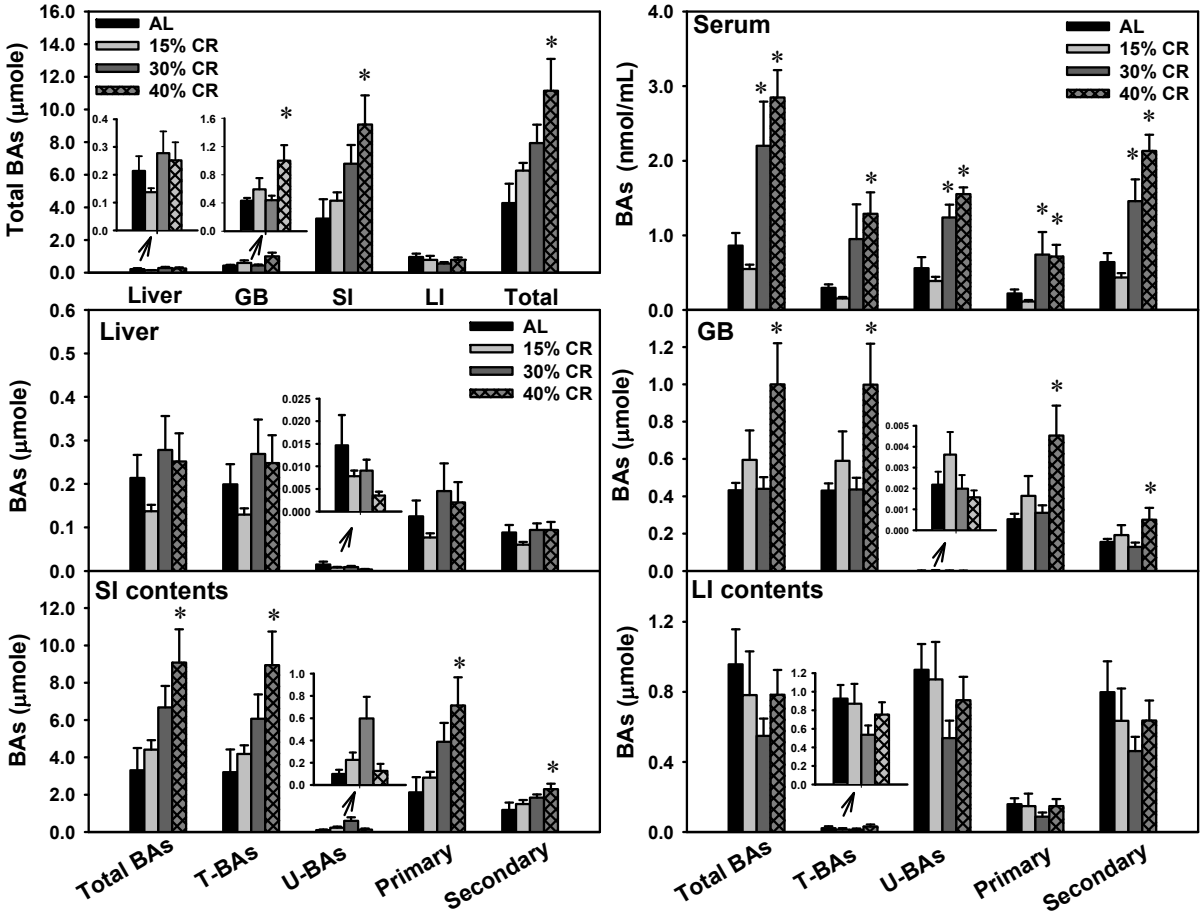
In serum **(Fig. 7.2, top-right panel)**, 40% CR increased total BA concentrations (231%). Specifically, 40% CR increased the concentrations of both conjugated (335%) and unconjugated BAs (178%). In addition, 40% CR increased similarly the concentrations of primary (224%) and secondary BAs (233%). In liver **(Fig. 7.2)**, CR did not alter the amounts of conjugated, unconjugated, primary, or secondary BAs. In GB **(Fig. 7.2)**, 40% CR increased the amounts of conjugated (132%), primary (162%), and secondary BAs (77%), however, CR did not alter the amounts of unconjugated BAs. In the SI contents **(Fig. 7.2)**, 40% CR increased the amounts of conjugated (179%), primary (219%), and secondary BAs (94%), however, CR did not alter the amounts of unconjugated BAs. In the LI contents **(Fig. 7.2)**, CR did not alter the amounts of conjugated, unconjugated, primary, or secondary BAs.

Fig. 7.1.



Body weight, glucose, and lipid parameters in CR mice. (A) Body weight changes of mice by graded CR (AL, 15%, 30%, or 40% CR) throughout the study. Day 0 represents the start for one-month CR. The body weights of CR mice started to be lower than AL mice after 1 week of CR feeding. (B) Glucose tolerance test (GTT). After 6 h of fasting, the basal blood glucose (time 0) was taken. Then a bolus of 20% D-glucose was given (i.p.) and serial glucose levels were taken at indicated time points thereafter. The blood glucose levels of CR mice were lower than AL mice after glucose challenge. In addition, 30 and 40% CR mice had lower blood glucose than AL mice before glucose challenge as well. (C) Triglycerides (TG), cholesterol, and fatty acids (FAs) in plasma and liver. Data are presented as means ± SEM of 5 mice. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test (not shown in A and B panels).

Fig. 7.2.



Effect of graded CR on total BAs in various compartments of the enterohepatic circulation. (Top left) Total BA amounts in liver, gallbladder (GB), contents of small intestine (SI) and large intestine (LI), as well as total BA pool size. (Top right) Concentrations of total, taurine-conjugated (T-BAs), unconjugated (U-BAs), primary, and secondary BAs in serum. (Bottom) Amounts of total, conjugated, unconjugated, primary, and secondary BAs in liver, GB, SI, and LI. Data are presented as means ± SEM of 5 mice. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test.

7.3.3 Concentrations of Individual BAs in Various Compartments of CR Mice.

Concentrations of Individual BAs in Serum of CR Mice. Four BA pathways are classified to aid in reading **Fig. 7.3**, each of which involves the primary BA and its secondary BAs, as well as their taurine conjugates. For example, the CA pathway involves TCA, TDCA, CA, and DCA. In serum, 40% CR markedly increased the concentrations of TCA (872%), TDCA (11.2-fold), and DCA (10.6-fold) **(Fig. 7.3)**. CA was increased by 30% (262%), but not by 40% CR. For the CDCA pathway, CR did not alter the concentrations of TCDCA, TLCA, TUDCA, or CDCA in serum, but LCA was increased by both 30% (262%) and 40% CR (408%), whereas, UDCA was increased by 30% (205%), but not 40% CR. For the αMCA pathway, 30% CR increased concentrations of TαMCA (558%) and αMCA (489%), whereas 40% CR did not alter either. The secondary BAs MDCA and TMDCA were not altered by CR. For the βMCA pathway, CR did not alter the concentrations of BA taurine conjugates (TβMCA, TωMCA, or THDCA) or βMCA. The concentration of ωMCA was increased by both 30% (95.6%) and 40% CR (135%), as was HDCA (128% and 169%).

Amounts of Individual BAs in Livers and Gallbladders of CR Mice. In livers **(Fig. 7.3)**, for the CA pathway, 30 and 40% CR tended to double the amount of TCA in livers (not statistically different). TDCA amount was increased by both 30% (222%) and 40% CR (484%). CR did not alter CA amount. For the CDCA pathway, 40% CR decreased the amounts of TCDCA (45.9%), TUDCA (57.2%), but increased CDCA (146%). CR did not alter the amounts of TLCA or UDCA in liver. For the αMCA pathway, CR did not alter the amounts of BAs (TαMCA, TMDCA, αMCA, or MDCA) in

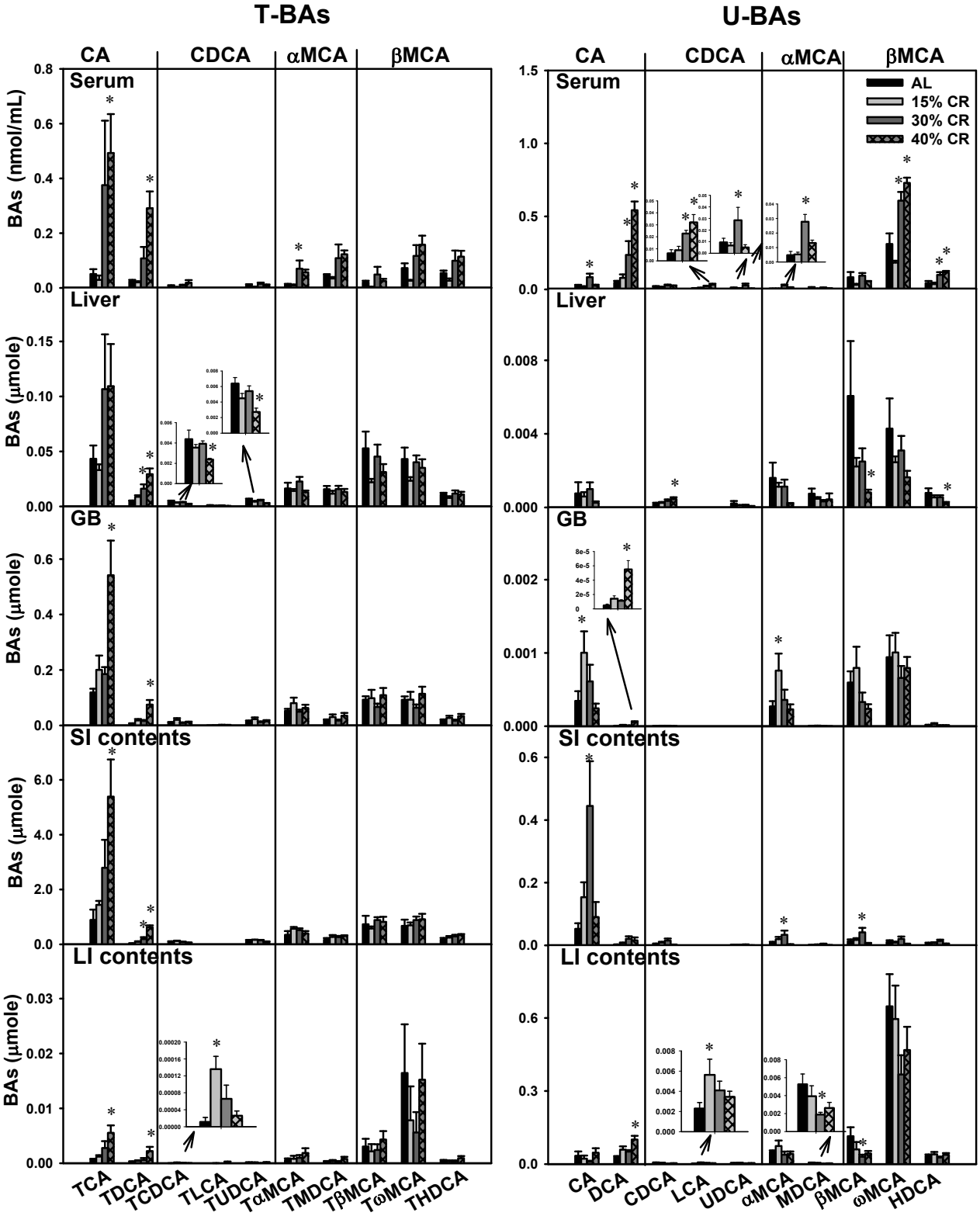
liver. For the βMCA pathway, 40% CR decreased the amounts of βMCA (86.6%) and HDCA (70.0%), but CR did not alter the amounts of TβMCA, TωMCA, THDCA, or ωMCA.

In gallbladders (GBs) **(Fig. 7.3)**, for the CA pathway, 40% CR increased the amounts of TCA (355%), TDCA (10.4-fold), and DCA (10.4-fold), and 15% CR increased CA amount (190%). CR did not alter the amounts of BAs in the CDCA, αMCA, or βMCA pathways, except a modest increase in αMCA by 15% CR (180%).

Amounts of Individual BAs in Intestinal Contents of CR Mice. In the contents of the small intestines (SI) **(Fig. 7.3)**, for the CA pathway, 40% CR increased the amounts of TCA (506%) and TDCA (18.0-fold), 30% CR increased CA amount (746%), whereas CR did not alter DCA amount. CR did not alter the amounts of BAs in the CDCA, αMCA, and βMCA pathways, except modest increases in αMCA (137%) and βMCA (210%) by 30% CR.

In the contents of the large intestines (LI) **(Fig. 7.3)**, for the CA pathway, 40% CR increased the amounts of TCA (694%), TDCA (639%), and DCA (253%), but CR did not alter CA amount. CR did not alter the amounts of BAs in the CDCA pathway, except the increases in TCDCA (694%) and LCA (145%) by 15% CR. CR did not alter the amounts of BAs in the αMCA or βMCA pathways, except decreases in MDCA (64.2%) and βMCA (73.6%) by 30% CR.

Fig. 7.3.


T-BAs
U-BAs
CA
CDCA
αMCA
βMCA
Serum
Liver
GB
SI contents
LI contents
BAs (nmol/mL)
BAs (μmole)
AL
15% CR
30% CR
40% CR
TCA
TDCA
TCDCA
TLCA
TUDCA
TαMCA
TMDCA
TβMCA
TωMCA
THDCA
CA
DCA
CDCA
LCA
UDCA
αMCA
MDCA
βMCA
ωMCA
HDCA

Continued to the following page.

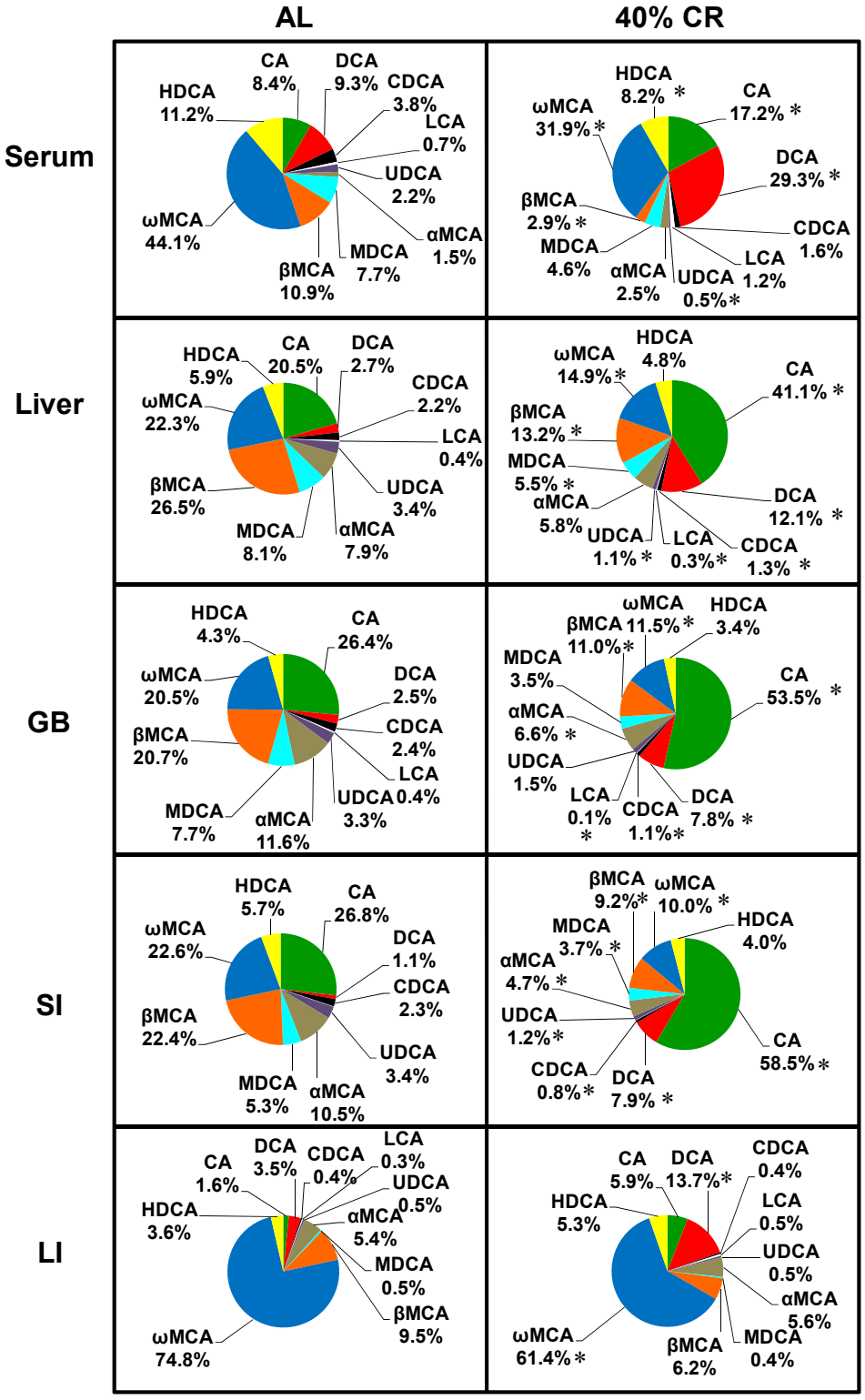
Fig. 7.3. (continued)

Individual BAs in various compartments of the enterohepatic circulation in CR mice. Four pathways are classified, and each (CA pathway for instance) involves primary BAs (CA), and secondary BAs (DCA), and their taurine conjugates (TCA and TDCA). CA, cholic acid; DCA, deoxycholic acid; CDCA: chenodeoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; MCA, muricholic acid; MDCA, murideoxycholic acid; HDCA, hyodeoxycholic acid. Data are presented as means ± SEM of 5 mice. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test.

7.3.4 Altered Enterohepatic BA Composition by 40% CR.

BA compositions in AL and 40% CR groups were analyzed by calculating the proportion of each BA and its taurine conjugates in total BA concentrations. In general, 40% CR increased the proportions of BAs in the CA pathway, but decreased the proportions of BAs in the CDCA, αMCA, and βMCA pathways **(Fig. 7.4)**. Specifically, 40% CR doubled CA proportions in serum (8.4% to 17.2%), liver (20.5% to 41.1%), GB (26.4% to 53.5%), and SI (26.8% to 58.5%), and tended to increase CA proportion in LI (1.6% to 5.9%; not statistically different). Similarly, 40% CR increased DCA proportions in serum (9.3% to 29.3%), liver (2.7% to 12.1%), GB (2.5% to 7.8%), SI (1.1% to 7.9%), and LI (3.5% to 13.7%). In contrast, 40% CR decreased βMCA proportions in serum (10.9% to 2.9%), liver (26.5% to 13.2%), GB (20.7% to 11.0%), and SI (22.4% to 9.2%). In addition, 40% CR decreased ωMCA proportions in serum (44.1% to 31.9%), liver (22.3% to 14.9%), GB (20.5% to 11.5%), SI (22.6% to 10.0%), and LI (74.8% to 61.4%).

Fig. 7.4.


AL
40% CR
Serum
Liver
GB
SI
LI
CA 8.4%
DCA 9.3%
CDCA 3.8%
LCA 0.7%
UDCA 2.2%
αMCA 1.5%
MDCA 7.7%
βMCA 10.9%
ωMCA 44.1%
HDCA 11.2%
HDCA 8.2% *
CA 17.2% *
DCA 29.3% *
CDCA 1.6%
LCA 1.2%
UDCA 0.5%*
αMCA 2.5%
MDCA 4.6%
βMCA 2.9% *
ωMCA 31.9%*
HDCA 5.9%
CA 20.5%
DCA 2.7%
CDCA 2.2%
LCA 0.4%
UDCA 3.4%
αMCA 7.9%
MDCA 8.1%
βMCA 26.5%
ωMCA 22.3%
HDCA 4.8%
CA 41.1% *
DCA 12.1% *
CDCA 1.3% *
LCA 0.3%*
UDCA 1.1% *
αMCA 5.8%
MDCA 5.5% *
βMCA 13.2% *
ωMCA 14.9%*
HDCA 4.3%
CA 26.4%
DCA 2.5%
CDCA 2.4%
LCA 0.4%
UDCA 3.3%
αMCA 11.6%
MDCA 7.7%
βMCA 20.7%
ωMCA 20.5%
ωMCA 11.5%*
HDCA 3.4%
CA 53.5% *
DCA 7.8% *
CDCA 1.1%*
LCA 0.1% *
UDCA 1.5%
αMCA 6.6% *
MDCA 3.5%
βMCA 11.0%*
HDCA 5.7%
CA 26.8%
DCA 1.1%
CDCA 2.3%
UDCA 3.4%
αMCA 10.5%
MDCA 5.3%
βMCA 22.4%
ωMCA 22.6%
βMCA 9.2%*
ωMCA 10.0% *
HDCA 4.0%
CA 58.5%*
DCA 7.9% *
CDCA 0.8%*
UDCA 1.2%*
αMCA 4.7% *
MDCA 3.7% *
LCA 0.3%
UDCA 0.5%
αMCA 5.4%
MDCA 0.5%
βMCA 9.5%
ωMCA 74.8%
HDCA 3.6%
CA 1.6%
DCA 3.5%
CDCA 0.4%
CA 5.9%
DCA 13.7%*
CDCA 0.4%
LCA 0.5%
UDCA 0.5%
αMCA 5.6%
MDCA 0.4%
βMCA 6.2%
ωMCA 61.4%*
HDCA 5.3%

Continued to the following page.

Fig. 7.4. (continued)

Altered enterohepatic BA composition by 40% CR. For instance, the green portion of the pie chart represents the proportion of CA and TCA in total BA concentrations. Data are presented as means of 5 mice. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test.

7.3.5 Expression of BA-Synthetic and Conjugating Enzymes in CR Mice.

The expression of BA synthetic and conjugating enzymes were quantified in the present study **(Fig. 7.5)**. In the classic pathway, 40% CR increased the mRNA of rate-limiting BA-synthetic enzyme Cyp7a1 (228%), but decreased the mRNA of Cyp8b1 (53.8%). In the alternative pathway, CR did not alter Cyp27a1 mRNA level. However, Cyp7b1 was decreased by 40% CR (76.2%), and Cyp39a1 was increased by 40% CR (440%). For BA conjugating enzymes, 40% CR increased BAL mRNA (123%), but CR had little or no effect on BAT mRNA level.

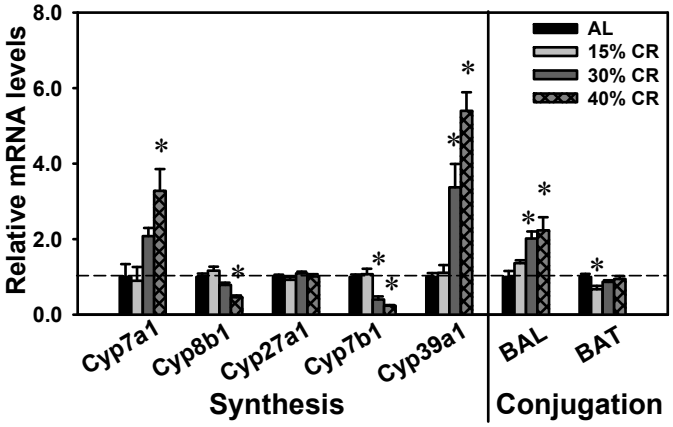
7.3.6 Expression of Cyp7a1 Transcription Regulatory Factors in Liver and Ileum of CR Mice.

The mRNAs of several key Cyp7a1 transcription regulatory factors were quantified **(Fig. 7.6)**. Forty percent CR increased the mRNAs of FXR in liver (87.5%) and SHP in ileum (120%). In addition, 40% CR tended to increase the mRNA of Fgf15 in ileum, but was not statistically increased. CR did not alter the mRNA levels of other Cyp7a1 transcription regulatory factors, such as SHP and Fgfr4 in liver, and FXR in ileum.

7.3.7 Expression of BA Transporters in the Enterohepatic Circulation in CR Mice.

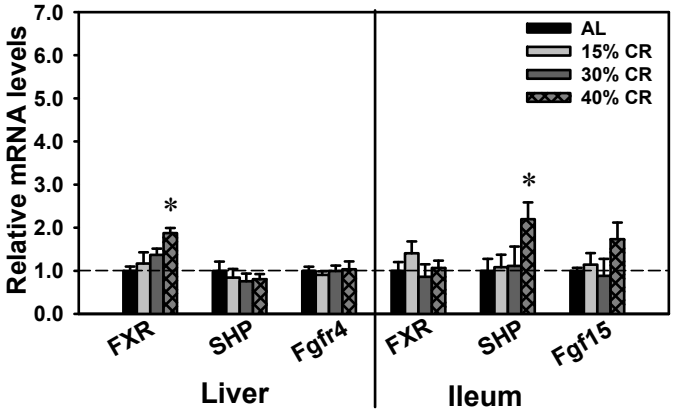
The expression of BA transporters in liver and ileum was quantified in the present study **(Fig. 7.7)**. Forty percent CR increased the mRNAs of Ntcp (35%) in liver and Ibabp (206%) in ileum. However, CR did not alter the mRNA levels of other BA transporters, including Oatp1b2 and Bsep in liver, and Asbt, Ostα, and Ostβ in ileum.

Fig. 7.5.



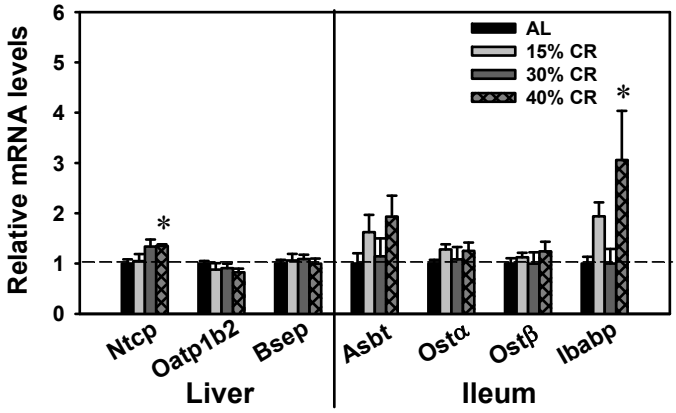
Expression of BA-synthetic and conjugating enzymes in CR mice. Cyp7a1 and Cyp8b1 are enzymes in the classic pathway for BA synthesis. Cyp7a1 is the rate-limiting enzyme for BA biosynthesis. Cyp27a1, Cyp7b1, and Cyp39a1 are enzymes in the alternative pathway for BA synthesis. BAL and BAT are BA conjugating enzymes. Data are presented as means ± SEM of 5 mice. The mRNA levels of each genes in AL mice were controls, set as 1. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test.

Fig. 7.6.



Expression of Cyp7a1 transcription regulatory factors in liver and ileum of CR mice. The transcription of Cyp7a1 is regulated by BA-induced feedback inhibition. One proposed mechanism involves BA receptor FXR and its target gene nuclear receptor SHP in liver. The other mechanism involves "enterohepatic communication", where BAs activate FXR in the intestine, inducing an intestinal hormone Fgf15, which travels through the circulation to the liver and through Fgfr4 down-regulates Cyp7a1 transcription. Data are presented as means ± SEM of 5 mice. The mRNA levels of each genes in AL mice were controls, set as 1. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test.

Fig. 7.7.



Expression of BA transporters in the enterohepatic circulation in CR mice. In liver, BA uptake transporters Ntcp extracts conjugated BAs and Oatp1b2 extracts unconjugated BAs into hepatocytes, and the BA efflux transporter Bsep pumps out BAs into canalicular bile. In ileum, the BA uptake transporter Asbt mediates the reabsorption of BAs from the intestinal lumen into ileocytes, the BA binding protein Ibabp mediates the intracellular transfer of BAs from apical to basolateral side of ileocyte membrane, and the BA efflux transporter Ostα/β pumps BAs out into portal blood. Data are presented as means ± SEM of 5 mice. The mRNA levels of each genes in AL group were controls, set as 1. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test.

7.4 Discussion

BAs are proposed to be potential mediators of longevity (Amador-Noguez et al., 2007; Gems, 2007; Goldberg et al., 2010a). The main hypothesis is that BAs could up-regulate xenobiotic metabolism and thus decrease molecular damage by toxic lipophilic by-products, which is a major contributor to aging (Gems and McElwee, 2005). The present study for the first time investigated the regulation of BA homeostasis by CR, the best known dietary intervention to ensure longevity, and comprehensively characterizes the BA profiles by CR in various compartments of the enterohepatic circulation. Using a graded CR model, the present study demonstrates coordinated increases in BA pool size as well as BA concentrations/amounts by 40% CR in various compartments of the body, which correlates with the up-regulation of BA-synthetic and conjugating enzymes and ileal BA binding protein.

Interestingly, graded CR increases several individual BAs in a "calorie-dependent" manner **(Fig. 7.3)**. It appears that CA and its secondary BA (DCA) have the highest inverse correlation to calorie intake. In general, the secondary BAs (such as DCA, LCA, ωMCA, and HDCA) increase more than primary BAs after CR. The present finding that some BAs such as CA, DCA, and LCA are increased by CR is consistent with previous reports that indicate that BAs might be longevity signals (Gems, 2007). The long-lived lit/lit mice have increased concentrations of several BAs in serum, such as LCA, DCA, CDCA, CA, etc. Moreover, feeding CA to wild-type mice reproduced the expression profiles of xenobiotic metabolism genes observed in the long-lived mice (Amador-Noguez et al., 2007). Increased resistance to stress and thus decreased

age-related damage to macromolecules such as DNA, lipids, and proteins have been proposed as a possible anti-aging mechanism (Gems and McElwee, 2005). In addition, LCA can increase the life span of yeast in a chemical screen assay (Goldberg et al., 2010b). These studies provide some indication that BAs might be "the fountain of youth" (Ferbeyre, 2010a). Therefore, the present study provides further support for this theory by demonstrating that CR, the most effective dietary intervention to retard aging, increases BA concentrations/amounts in several compartments of the EHC.

The CR-induced increase in BAs would not be expected to cause hepatotoxicity. For example, in the bile duct ligation-induced cholestatic liver injury model, total BA concentrations increased 1,400-fold in serum at 6 h after surgery (Zhang et al., 2012a). In comparison, the increase in total BA concentrations in serum by CR (2.3-fold) in the present study is hundreds of magnitudes lower **(Fig. 7.2)**. Feeding DCA to mice (0.1% in diet for 7 days) leads to a 19-fold increase in DCA concentration in serum, and the serum alanine aminotransferase activity is about 50 IU/L (Song et al., 2011), indicating little or no hepatotoxicity. Smaller increases in BA concentrations/amounts are identified in the present study, with the highest increase in TDCA, which is increased about 11-fold in serum, 5-fold in liver, 10-fold in GB, 18-fold in SI, and 6-fold in LI. Thus, the CR-induced BA increases do not appear to have reached the concentration to cause toxicity.

Furthermore, CR increases 12-hydroxylated BAs in various compartments of the EHC. CR alters BA composition **(Fig. 7.4)**, namely increasing the proportions of CA and DCA, and decreasing the proportions of α-, β-, and ωMCAs. Both CA and its secondary

BA, DCA, have 12-hydroxyl groups, whereas the MCAs do not. In addition to the increased proportions, the concentrations/amounts of CA and DCA are increased in most compartments as well **(Fig. 7.3)**. Our finding on the correlation of increased proportions of 12-hydroxylated BAs and improved insulin sensitivity and decreased lipid parameters **(Fig. 7.1B and C)** in CR mice is consistent with the previous report that impaired generation of 12-hydroxylated BAs was associated with dyslipidemia and insulin resistance (Haeusler et al., 2012). Therefore, our study provides further evidence for the role of BA composition or the presence of certain class of BAs in the regulation of glucose and lipid metabolism.

The increases in BAs by CR correlate with the alterations of genes related to BA homeostasis. Cyp7a1 plays the leading role in determining BA synthesis and BA pool size (Li et al., 2011). In the present study, 40% CR increases Cyp7a1 expression **(Fig. 7.5)**, which correlates with the increase of BA pool size **(Fig. 7.2)**. This suggests that the increases in BA pool size by CR are likely due to increased expression of Cyp7a1. Opposite to the increase in CA, the expression of Cyp8b1, the enzyme that forms CA, is down-regulated by 40% CR **(Fig. 7.5)**. Some BA synthetic enzymes have gender-divergent expression in liver. Both Cyp8b1 and Cyp7b1 are expressed higher in male than female mice (Fu et al., 2012b), which is opposite to Cyp39a1 (female > male) (Li-Hawkins et al., 2000a). Interestingly, the expression of Cyp8b1 and Cyp7b1 were decreased by CR, whereas Cyp39a1 was increased **(Fig. 7.5)**. This appears to be attributable to feminization of the liver by CR. Previous reports have shown that CR can feminize genome-wide gene expression in male mice (Estep et al., 2009) and

long-lived mice have lost the sex dimorphism of gene expression (Amador-Noguez et al., 2005). Therefore, the current finding of CR-induced feminization of some BA synthetic enzymes suggests that the connection between feminization and longevity is an interesting area of research.

Expression of the rate-limiting BA synthetic enzyme Cyp7a1 is regulated by BA-feedback inhibition through two mechanisms involving the liver FXR-SHP pathway and the intestinal FXR-Fgf15 pathway. Recent data suggest that FXR-SHP plays a minor role in suppressing Cyp7a1, but is more important in Cyp8b1 suppression, whereas Fgf15 is important for both Cyp7a1 and 8b1 regulation (Kim et al., 2007; Kong et al., 2012). The expression of the FXR target gene SHP in liver is not altered by CR **(Fig. 7.6)**, indicating that FXR activation by BAs in liver remains constant during CR. This correlates with the observation that CR does not alter the amounts of total BAs in liver **(Fig. 7.2)**. In contrast, the expression of SHP in ileum is increased by CR, and another FXR target, Fgf15, also tends to be increased **(Fig. 7.6)**, indicating that FXR activation in ileum is likely increased by CR, which may be due to increased the amounts of total BAs in SI **(Fig. 7.2)**. In summary, CR appears to increase FXR activation by BAs in intestine, and thus mediate feedback inhibition of Cyp8b1. However, little or no feedback inhibition of Cyp7a1 is observed, possibly because of intrinsic induction of Cyp7a1 expression by CR.

It is likely that CR increases BA conjugation in livers. The mRNA of the BA conjugating enzyme BAL is increased by 30% CR (101%) and 40% CR (123%) **(Fig. 7.5)**. This correlates with the increases in the proportions of taurine-conjugated BAs in

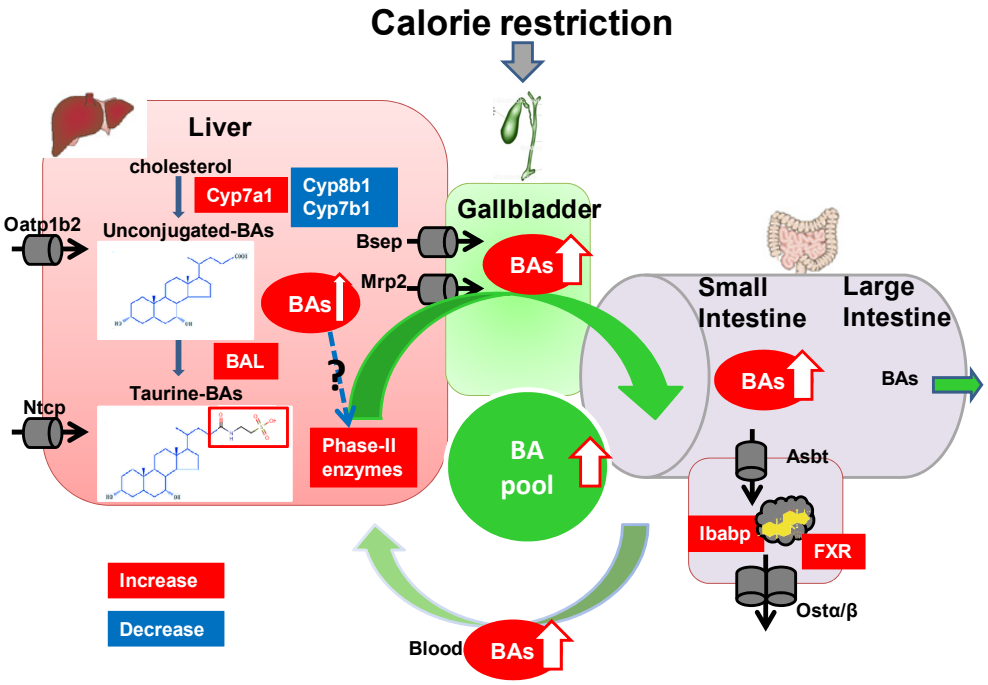
serum (34% to 45%), liver (93% to 98%), and SI contents (97% to 99%) **(Fig. 7.4)**. In addition, the proportions of conjugated and unconjugated forms of some individual BAs are altered differently by CR. For example, the concentration of CA is not altered by 40% CR, whereas TCA increases by 40% CR in serum (872%), livers (369%), GBs (432%), SI (506%) and LI contents (694%) **(Fig. 7.3)**. These differences are likely caused by an increase in the BA conjugating enzyme BAL by CR, and therefore, possibly increased taurine-conjugation of BAs.

Interestingly, 40% CR increases the expression of ileal BA binding protein Ibabp, which is thought to mediate intracellular BA transfer in ileocytes (Grober et al., 1999). This suggests that CR could possibly increase the efficiency of BA recycling from the intestine back to liver. In general, CR does not alter the expression of BA transporters in liver and ileum **(Fig. 7.7)**.

In conclusion, the present study demonstrates that CR increases BAs, possibly through orchestrated increases in BA synthesis and conjugation in liver and intracellular transport in ileum. This study provides new evidence to support the connections between BAs and longevity by using this CR model. Due to the difficulty to study endpoints of aging process in mammals, determining good biomarkers for aging and thus to evaluate anti-aging interventions is of great importance. Compared to the previous limited knowledge about the connection between BAs and longevity, the present study provides comprehensive information about the BAs that are increased by CR, which lays a foundation for future studies on BAs as potential biomarkers for longevity, and possibly the development of BA-like compounds as anti-aging drugs.

Chapter 8: CONCLUSIONS AND DISCUSSION

Fig. 8.1.



The regulation of BA and xenobiotic metabolism by CR. CR increases BA pool size, which is likely due to increased BA synthesis (Cyp7a1) and conjugation (BAL) as well as intestinal BA reabsorption (Ibabp). Amounts of total BAs are increased by CR in gallbladder and small intestine, but remain relatively constant in liver. Total BA concentrations in liver tend to double by CR, which may contribute to the CR-induced upregulation of phase-II xenobiotic metabolizing enzymes.

The present dissertation has characterized alterations of the expression of XPGs in liver and BA homeostasis by aging and the anti-aging intervention CR. This dissertation identified gender divergent age-dependent changes of the expression of XPGs and female-specific elevation of total BA concentrations in serum during aging, and demonstrated that CR can feminize the expression profile of many XPGs and increase BAs in male mice **(Fig. 8.1)**.

Chapter 4 comprehensively characterized the mRNA profiles of XPGs with important functions in metabolism and disposition in liver during aging. Nine groups of mice from 3 to 27 months of age were investigated, which provides much more detailed information of age-dependent changes than previous reports (Peng et al., 2005; Mori et al., 2007; Lee et al., 2008). About 40% of the XPGs (such as Oatp1a1, Cyp1a2, Ces1f, 2c, Sult5a1, Gstt2, Mrp3, and Bcrp) have lower expression in liver in old mice, which appears to correlate with decreased hepatic drug metabolism and disposition in the elderly (Bressler and Bahl, 2003; Schmucker, 2005). Using both male and female mice, the present study reveals novel gender-divergent mRNA profiles of XPGs across the lifespan. More XPGs with elevated expression with aging are observed in female (21 XPGs) than male (4 XPGs) mice. For instance, the increased expression of Nqo1, Sult1e1, 2a1/2, and Mrp4 are only observed in female mice. According to the detoxification theory of aging, up-regulation of XPGs renders broad spectrum detoxification and is proposed to be one of the best longevity assurance mechanisms. Females have a longer life expectancy than males in humans (Vina et al., 2005) and rodents (Goodrick, 1975; Harrison et al., 2009). Thus, the observations in Chapter 4

about the gender differences of XPG expression during aging appear to correlate with the phenomenon that females tend to live longer than males.

Chapter 5 demonstrates gender-divergent alterations of BA homeostasis during aging. Total BAs in liver are not altered during aging. Total BA concentrations in serum increase 340% from 3 to 27 months in female mice, but remain unchanged with age in male mice. Very recent reports suggest a connection between BAs and long life span (Gems, 2007). The observation in Chapter 5 of higher BA concentrations in serum in aged female mice than aged male mice correlates with the longer life span in females. Furthermore, the differences in BA concentration in serum between genders appear to be due to synergistic effects of female-specific increased expression of BA uptake transporters Ntcp and Oatp1b2 in liver as well as the rate-limiting enzyme for BA synthesis Cyp7a1.

After the effect of aging on XPG expression and BA metabolism had been determined, Chapter 6 investigated the regulation of XPG expression in liver by CR, the best anti-aging intervention in mammals. Previous reports on the regulation of XPGs in CR mice were very inconsistent, mainly due to the large variation of diets, feeding regimens, as well as age and strain of the mice. Therefore, the "dose-response" model of CR in Chapter 6 enables a reliable comparison under the same experimental conditions to identify how graded CR regulates the expression of XPGs in liver. Data show that CR up-regulates a large number of XPGs, such as uptake transporter Oatp1a4, phase-I enzymes Cyp4a14, Por, Nqo1, Fmo2, 3, and phase-II enzymes Sult1a1, 1d1, 1e1, 3a1, Gsta4, m32. m3, and Mgst3. These changes are consistent

with previous observations of the association of up-regulation of XPGs and increased life span in *C. elegans (*McElwee et al., 2004), *Drosophila* (Iqbal et al., 2009), as well as several long-lived mice (dwarf mice, Little mice, and rapamycin-treated mice) (Amador-Noguez et al., 2007; Steinbaugh et al., 2012). The present finding provides a critical piece of evidence in support of the detoxification theory of aging, which suggests increased detoxification of a broad spectrum of toxic metabolites is beneficial for longevity.

In addition to the up-regulation of many XPGs, another important finding in Chapter 6 is that CR appears to feminize the expression of over half of the XPGs that are altered. Previous studies have shown that CR feminizes gene expression profiles in livers of male mice (Estep et al., 2009) and mutation of GH/IGF-1 axis produced an almost complete loss of the sex-specific gene expression in livers of long-lived dwarf mice (Amador-Noguez et al., 2005). These conclusions were generated from genome-wide microarray studies, providing the global changes. Being unique in focusing on the xenobiotic metabolism pathway, Chapter 6 suggests that many gender-divergently expressed XPGs are feminized by CR, and the changes appear to be "dose-dependent" on the degree of CR. Taking the longer life expectancy in females than males into consideration, the CR-induced feminization effects on the expression of some XPGs in liver may be consistent with the beneficial effects of CR on longevity. Furthermore, decreased GH levels (Chacon et al., 2005) and IGF-1 signaling in CR mice (Barzilai and Bartke, 2009) may be the mechanism for the feminization of XPGs by CR, which is

similar to the finding in long-lived dwarf mice with deficient GH/IGF-1 signaling (Amador-Noguez et al., 2005).

However, exceptions exist because some XPGs that are altered by CR either do not have gender-divergent expression (such as Oat2, Ces1e, Ugt3a1, and Mrp3) or their changes by CR fail to be explained by feminization effects (such as Oct1, Ces1f, Sult5a1, and Mdr2). Some XPGs with gender-divergent expression are not changed by CR, such as Cyp2b10, Ugt1a9, and Mate1. The mechanisms for those changes need further investigation.

Chapter 7 represents the first study on the regulation of enterohepatic circulation of BAs by CR. Individual BAs were quantified by UPLC-MS/MS in various compartments of the enterohepatic circulation, including serum, liver, gallbladder, small and large intestine, in order to systematically investigate the regulation of BA homeostasis by CR. CR increases the BA pool size (162%) and total BA concentrations/amounts in serum, gallbladder, and small intestine, which may be attributable to the increased expression of BA-synthetic (Cyp7a1) and conjugating enzyme (BAL) as well as ileal BA-binding protein. The current finding of increased BAs by CR in mice is consistent with previous reports of the connections of BAs and longevity regulation in yeast (Goldberg et al., 2010a), *C. elegans* (Gerisch et al., 2007), and long-lived Little mice (Amador-Noguez et al., 2007). This evidence provides important support to the proposed roles of BAs as anti-aging molecules (Gems, 2007). Quantification of various individual conjugated and unconjugated BAs by UPLC-MS/MS technique in Chapter 7 reveals that graded CR "dose-dependently" increases the concentrations of many individual BAs in serum, such

as TCA, TDCA, DCA, LCA, ωMCA, and HDCA. Interestingly, many of these BAs are secondary BAs, which are generally thought to be more toxic than primary BAs due to increased hydrophobicity. It would be important to investigate whether this phenomenon has any functional significance. For instance, this may be an example of the hormesis theory where a low dose of "toxic" compounds can activate stress defense mechanisms that ensure longevity. This observation provides insight and clues to future directions of research and therapy, such as choosing some individual BAs as potential markers of aging and anti-aging process, and possibly developing BAs or BA-like compounds to increase longevity.

Chapter 7 also reveals interesting alterations of BA composition by CR. CR increases the proportions of BAs in the CA pathway (namely CA and DCA), and decreases the proportions of BAs in MCA pathway (namely α-, β-, and ωMCAs), which is exactly opposite to the BA composition changes observed during aging, which is increased proportion of βMCA and decreased proportion of DCA as shown in Chapter 7. This observation that CR regulates BA composition in an exactly opposite way to the regulation by aging suggests that the change in BA composition may be an overlooked marker for longevity regulation. One major difference of BAs in the CA pathway compared to those in MCA pathway is that both CA and DCA have 12α-hydroxyl groups. The observation of a correlation of increased proportions of 12-hydroxylated BAs and improved insulin sensitivity and decreased lipid parameters in CR mice in Chapter 6 are consistent with a previous report that impaired generation of 12-hydroxylated BAs was associated with dyslipidemia and insulin resistance (Haeusler et al., 2012). Therefore,

data in Chapter 7 provides important evidence for the role of BA composition (for instance the ratio of BAs in CA vs MCA pathways) in the regulation of glucose and lipid metabolism.

The novel role of BAs as potential anti-aging molecules has been shown in yeast (Goldberg et al., 2010a; Goldberg et al., 2010b), and more studies are needed to investigate whether BAs are effective in increasing life span in higher organisms. To address this question, BA feeding studies with life span as endpoints will provide important *in vivo* data. Mouse models are a reasonable start for research in mammals. BAs are natural detergents with amphypathic properties, and high concentrations of BAs are cytotoxic and associated with various liver toxicity and diseases, as well as colorectal cancer. Therefore, the dose of BA feeding is a critical aspect of the study design. A previous report showed 2% CA feeding for 7 days up-regulated a largely overlapping set (close to 80%) of the XPGs up-regulated in long-lived Little mice (Amador-Noguez et al., 2007). Although this model has value in addressing the sequential events of increased BAs, and then increased XPG expression that appear to mediate beneficial roles in longevity in Little mice, the study design of feeding CA at this high concentration across the life span with the expectation to observe increased longevity is likely to be inappropriate. This is because liver toxicity (ALT is about 500 IU/L) was induced by 1% CA feeding for 7 days in a previous BA feeding study in mice by our laboratory (Song et al., 2011). Various individual BAs have different potencies in activating BA receptors FXR and TGR5, as well as regulating gene expressions. Therefore, choosing correct individual BAs or combination of various BAs at the correct concentrations is of great

importance when performing BA feeding study across the life span in mice. Furthermore, life span studies in transgenic mice deficient or overexpressed with BA synthetic enzymes would provide valuable evidence for the possible role of BAs in longevity. In addition, possible hormesis mechanisms of BAs that extend life span through up-regulation of XPGs need to be investigated as well.

In summary, utilizing a "dose-response" model of CR, analytical tools, and molecular techniques, the present dissertation has investigated alterations of the expression of XPGs in liver and BA homeostasis with aging, and more importantly the regulation of XPG expression in liver, and the enterohepatic circulation of BAs by the best known anti-aging intervention, CR. The current findings of CR-induced up-regulation of many XPGs and elevation of BAs provide further evidence for the detoxification theory of aging, and promote understanding the possible role of BAs as longevity signaling molecules, and also provide useful information on drug elimination pathways that one should monitor in the elderly as well as people on diets.

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