MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's thesis

„Changes in bile acid levels in response to caloric restriction and modulation of the caloric restriction-related pathways in mice“

verfasst von / submitted by

Gabriele Benischke, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2022 / Vienna, 2022

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 838
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Ernährungswissenschaften UG2002
Betreut von / Supervisor:	Dr. techn. Kalina Duszka

Acknowledgement

First of all, I would like to thank András, who was always available to answer my questions, and they were certainly not a few. No matter if I needed help with the LCMS, the laboratory devices, or my thesis, he was always there to answer my questions. Thanks!

Next, I want to thank my supervisor Kalina, who supported me with lots of professional expertise and always was there to answer my question. Moreover, she has always kept me motivated to finish this thesis!

Special thanks go to my family and my boyfriend who supported me throughout my whole study.

Last but not least, I want to thank my study colleagues who worked together with me in the laboratory and always made working on this thesis much more fun. Thanks to Kathrin, Stefan, Alex, and Simon, who always had an ear open for my questions.

Abbreviation list

µl	Mikro litres
ΔΔCt	Delta delta Ct
ABCA1	ATP-binding cassette transporter ABCA, member 1
ABCG1	ATP-binding cassette transporter ABCG, member 1
ABCG5	ATP-binding cassette, subfamily G (white), member 5
ACN	Acetonitrile
AT	Antibiotics
AUC	Area under the curve
BA	Bile acid
BAAT	Bile acid CoA-Amino acid N-acetyltransferase
BCAT	Bile acid CoA synthase
BIOS2	Biospherian 2
°C	Celsius
C	Cellulose Bedding
C10	Capric acid
C8	Caprylic acid
CA	Cholic acid
CC	Corncob Bedding
CDCA	Chenodeoxycholic acid
CKD	Classical ketogenic diet
CR	Caloric restriction
CR-C	Caloric restriction cellulose bedding
CR-CC	Caloric restriction corncob bedding
CR-W	Caloric restriction wooden bedding
Ct	Cycle threshold
CYP2B	Cytochrome P450 2B
CYP2C	Cytochrome P450 2C
CYP3A4	Cytochrome P450 3A4

CYP7A1	Cholesterol 7 alpha-hydroxylase/ Cytochrome P450 7A1
CYP8B1	Sterol 12-alpha-hydroxylase/ Cytochrome P450 8B1
CYP27A1	Sterol 27-hydroxylase/ Cytochrome P450 27A1
DCA	Deoxycholic acid
E	Empty
EC	Enterohepatic circulation
ESI	Electrospray ionisation
EtOH	Ethanol
eWAT	Epididymal White adipose tissue
FGF15	Fibroblast growth factor 15
FGF19	Fibroblast growth factor 19
FGF21	Fibroblast growth factor 21
FGFR4	Fibroblast growth factor receptor 4
FMD	Fasting mimicking diet
FT	Faecal transplant
FXR	Farnesoid X-receptor
G	G-Force (setting for centrifuge)
g	Gram
G	Grid
GLP-1	Glucagon-like peptide-1
HLPC	High-Performance Liquid Chromatography
IBAT	Ileal bile acid transporter
IF	Intermittent fasting
IGF-1	Insulin-like growth factor 1
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
Kcal	Kilocalorie
KD	Ketogenic diet
KO	Knock-out
LC	Liquid chromatography

LCA	Lithocholic acid
LCMS	Liquid chromatography mass spectrometry
LDL	Low-density lipoprotein
LGIT	The low glycaemic index treatment
LHR-1	Liver receptor homolog-1
LPS	Lipopolysaccharide
LTD	Low taurine diet
LXRα/β	Liver X-receptor alpha/beta
m/z	Mass to charge ratio
MAD	Modified Atkins diet
MCA	Muricholic acid
MCT	Medium chain triglyceride
MeOH	Methanol
Mg	Milligram
Min	Minutes
MM	Master mix
MRP2	Multidrug resistance-associated protein 2
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Ngn3	Neurogenins
NK-cells	Natural killer cells
NS	No significance
NTD	Normal taurine diet
OATP2	Solute carrier organic anion transporter family member 1B1
OH	Hydroxyl group
ON-W	Overnight fasting with wooden bedding
Ostα/β	Organic solute transporter subunit alpha/beta
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E2

PKA	Protein kinase A
PPARα	Peroxisome proliferator activated receptor alpha
PXR	Pregnancy x-receptor
PYY	Peptide YY
qPCR	Qualitative Polymerase Chain Reaction
RG	Reference gene
RP	Reverse phase
rpm	Rounds per minute
SHP	Small heterodimer partner
SI	Small intestine
SOX2	Sex determining region Y-box 2
SREBP1c	Sterol regulatory element binding protein 1c
STD	Standard
T1D	Diabetes type 1
T2D	Diabetes type 2
Tau	Taurine diet
TCA	Taurocholic acid
TDCA	Taurodeoxycholic acid
TF	Transcription factor
TG	Triglyceride
TGR5	Takeda G-protein receptor 5
TLCA	Taurolithocholic acid
TNF-α	Tumour necrosis factor alpha
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
W	Wooden Bedding

Table of content

List of tables

List of figures

Abstract

Caloric restriction (CR) is a dietary intervention involving reduced energy intake without causing malnutrition and is well-known for its health-promoting effects.

This thesis aims to verify how CR influences the bile acid (BA) metabolism and which pathways are included in the changes. Different types of cage beddings were applied to investigate the effect of fibre on BAs during CR in mice. To see whether the microbiome influences the increase of BA concentrations, antibiotics were dosed on mice. Additionally, a faecal transplant (FT) with the microbiota of CR or *ad libitum* (ad-lib) mice was performed. Moreover, the supplementation of taurine was used to access its impact on BA levels. Besides CR, the effects of other diets on BAs were studied. Intermittent fasting (IF), fasting-mimicking diet (FMD) and types of ketogenic diets (KD) were part of the experiment. IF is a form of fasting in which food is abstained for a certain period, FMD mimics the effects of fasting, and KD is a diet form which relies on fat as a source of energy.

For measuring the BA concentrations, Liquid Chromatography Mass Spectrometry was used. qPCR was applied to quantify genes involved in changes in the BA metabolism including transporters, receptors, and transcriptional factors.

Increased concentrations of BAs were measured in the ileum, the liver and plasma of CR compared to ad-lib mice. Moreover, genes that increase BA synthesis were upregulated during CR, whereas genes that inhibit the synthesis were downregulated. If mice consumed more fibre, CR mice still did not excrete more BAs via faeces. Cellulose in bedding led to the biggest BA increase in ileum and plasma. Taurine supplementation in ad-lib mice increased BA concentrations in the ileum and liver, however, CR mice without taurine had higher BA concentrations. After gut bacteria depletion fewer BAs were synthesised, and no secondary BAs were produced.

IF and FMD also increased BA concentrations in the ileum. In contrast, KD could not increase BA concentrations.

This thesis has shown, that under fasting conditions mice increase the production of BAs in the hepatocytes and reduce excretion via faeces. Based on these results we assume that more lipids

and fat-soluble vitamins can be absorbed and compensate for the reduced energy intake during CR.

Zusammenfassung

Kalorienrestriktion (CR) ist eine diätetische Maßnahme, bei der die Energiezufuhr reduziert wird, ohne dass es zu einer Unterernährung kommt, und die für ihre gesundheitsfördernde Wirkung bekannt ist.

In dieser Arbeit soll überprüft werden, wie CR den Gallensäuren (BA) -Stoffwechsel beeinflusst und welche Stoffwechselwege an den Veränderungen beteiligt sind. Es wurden verschiedene Arten von Käfigeinstreu verwendet, um die Auswirkungen von Ballaststoffen auf den BA-Stoffwechsel während der CR bei Mäusen zu untersuchen. Um zu sehen, ob das Mikrobiom den Anstieg der BA-Konzentrationen beeinflusst, wurden den Mäusen Antibiotika verabreicht. Zusätzlich wurde eine Fäkaltransplantation (FT) mit dem Mikrobiom von CR- oder ad libitum (ad-lib) Mäusen durchgeführt. Darüber hinaus wurde eine Taurin-Supplementierung durchgeführt, um die Auswirkungen auf den BA-Spiegel zu untersuchen. Neben CR wurden auch die Auswirkungen anderer Diäten auf die BAs untersucht. Intermittierendes Fasten (IF), Scheinfasten/ Fasting-Mimicking-Diet (FMD) und ketogene Diäten (KD) waren Teil des Experiments. IF ist eine Form des Fastens, bei der über einen bestimmten Zeitraum auf Nahrung verzichtet wird, FMD ahmt die Auswirkungen des Fastens nach, und KD ist eine Diätform, die auf Fett als Energiequelle setzt.

Zur Messung der BA-Konzentrationen wurde die Flüssigchromatographie-Massenspektrometrie verwendet. qPCR wurde zur Quantifizierung von Genen eingesetzt, die an Veränderungen im BA-Stoffwechsel beteiligt sind, darunter Transporter, Rezeptoren und Transkriptionsfaktoren.

Bei CR-Mäusen wurden im Vergleich zu ad-lib-Mäusen eine erhöhte BA-Konzentrationen im Ileum, in der Leber und im Plasma gemessen. Außerdem wurden Gene, die die BA-Synthese erhöhen, bei CR hochreguliert, während Gene, die die Synthese hemmen, herunterreguliert wurden. Auch wenn die Mäuse mehr Ballaststoffe zu sich nahmen, scheiden CR-Mäuse nicht mehr BAs über den Kot aus. Zellulose in der Einstreu führte zum größten BA-Anstieg im Ileum und im Plasma. Eine Taurin-Supplementierung bei ad-lib-Mäusen erhöhte die BA-Konzentrationen im Ileum und in der Leber, CR-Mäuse ohne Taurin hatten jedoch höhere BA-Konzentrationen. Nach der Darmbakteriendepletion wurden weniger BAs synthetisiert, und keine sekundären BAs gebildet.

IF und FMD erhöhten auch die BA-Konzentrationen im Ileum. Im Gegensatz dazu konnte KD die BA-Konzentrationen nicht erhöhen.

Diese Arbeit hat gezeigt, dass Mäuse unter Fastenbedingungen die Produktion von BAs in den Hepatozyten erhöhen und die Ausscheidung über die Fäkalien reduzieren. Aufgrund dieser Ergebnisse gehen wir davon aus, dass mehr Lipide und fettlösliche Vitamine aufgenommen werden können, um die reduzierte Energiezufuhr während der CR zu kompensieren.

1 Introduction

1.1 Caloric restriction (CR)

CR is a nutritional intervention with reduced energy intake but without malnutrition. It is the most studied robust non-genetic and non-pharmaceutical intervention in multiple animal models concerning the extension of health- and lifespan. For example, budding yeast, worms, and fruit flies but also mice and rats have shown an increase in lifespan if calorically restricted. The optimum reduction for mice and rats for the prevention or delay of many chronic diseases is a reduction between 20%-50%. Observations and clinical trials indicate that CR induces the same metabolic and molecular changes in humans as in animals (1).

1.1.1 History

For more than 500 years it is assumed that CR benefits the health and lifespan. The Italian nobleman Luigi Cornaro, lived beyond 80 in the 16th century, at a time when adult life expectancy was low (2,3). He held his dietary habits, “eating as little as possible” responsible for its health benefits (3). Another example, Sir William Temple (1628-1699), had an interesting approach. He described good health and longevity as a premium of the poor, and not the rich since being reliant on temperance is better for health than being dependent on excess. In other words, overconsumption is not beneficial for health (3).

McCay published one of the first experimental studies with animals, that are restricted to food and has made an impressive contribution to this field. First, he tried CR on fish, then on rats and concluded that 40% CR from the age of weaning extended the life span in rats dramatically. So did Osbourne et al. who showed that CR rats increased lifespan and reproductive performance (3–5).

1.1.2 Lifespan and mortality

It has been shown that there is a linear relationship between CR and mean and maximum lifespan in small rodents (3,6). The best life-extending effect can be observed if rodents start CR during weaning (3). Moreover, also starting at mid-adulthood, which is 12 months, can

extend the maximum lifespan (6). However, starting CR at an advanced age tends to increase the mortality rate. Age as well as the genotype of the mouse are independent determinants regarding the effects on lifespan (7).

Besides genotype and the beginning of CR, the extent of CR is also very important. The increase in life expectancy goes hand in hand with the degree of restriction, but not endlessly. The maximum effect seems to be achieved in animals receiving 40-45% less than the usual baseline intake. Restricting more than this leads to a negative effect on the lifespan of animals (3).

1.1.3 Further health aspects of CR

One of the main health aspects of CR is the protective effect on cancer (3). It contains the reduction of initiation, promotion as well as the progression of spontaneous tumours in several tissues (3,8). Excessive adiposity, unhealthy diets and less physical activity are the key players in cancer pathogenesis. A reduction of 15%-53% calorie intake in rodents has been shown a proportional linear reduction, 20%-62%, in tumour incidence. Some cancer types show a greater response to the effect of CR than others (8). Especially the positive effect of CR on breast cancer can be highlighted (9) but also other cancer types such as liver (10), skin (11), colon (12), pituitary (13) and the lymph system can be traced back to the positive effects of CR (3).

Positive effects can be attributed to markers associated with malignancies, which are reduced during CR. Circulating levels of growth factors, anabolic hormones, inflammatory cytokines and oxidative stress are diminished (8). Moreover, body composition plays a role as well. The reduced size of the lean tissue and less body fat are possible explanations for the protective cancer effects. Lowered leptin levels, as well as increased adiponectin levels during CR, are further explanations (3,14).

In the course of ageing, there is an increase in metabolic disorders, leading to hyperinsulinemia, insulin resistance, dyslipidaemia, and visceral obesity. These abnormalities were reversed in

rats, which were calorically restricted by 30% for three months. Especially reversing insulin resistance is a key mediator for increased lifespan (3).

The leading cause of age-related morbidity and mortality is atherosclerosis and cardiovascular disease (15,16). Studies indicate that CR delays cardiac ageing and can prevent cardiovascular disease (17–20). Furthermore, CR helped to reduce total serum cholesterol, low-density lipoprotein-cholesterol, triglycerides, fasting glucose and fasting insulin levels in a human experiment. Additionally, systolic and diastolic blood pressure was decreased (17). Apart from that, CR has a protective effect against cardiomyocytes by reducing DNA damage, enhancing DNA repair, inducing apoptosis-inhibitory genes and reducing the expression of pro-apoptotic genes (21). Moreover, the IMT (carotid artery intima-media thickness) was measured among participants in a study and was 40% less in the CR group (18). These findings suggest that CR is effective in reducing the risk of developing atherosclerosis (3,18).

Concerning the immune system, the effects of CR are nebulous. Studies have focused on the effects on T-lymphocytes and macrophage population, which are part of the adaptive immune system and also on natural killer cells (NK cells), part of the innate immune system (3). Data suggests that CR delays the maturation of the immune system in mice (22), and it showed positive effects by delaying the thymic involution (23,24). Thus, a greater T-lymphocyte production and activity in the later life of mice is attributable to CR. Besides, a marked reduction of autoimmune disease was observed (3,25). Moreover, CR mice had a greater immune capacity against a virus they had been vaccinated against (26). Concerning non-human primates, the effects are quite unclear and possibly a late start of CR will lead to fewer positive benefits. Precisely, an onset in early or late adulthood could even harm T-cell function. It is assumed that during adulthood an optimal window can delay the ageing of the immune system (27,28).

One major cellular impact of CR is to reduce oxidative stress (3,29,30). This can be attained via 3 steps: a reduction of the oxygen free-radicals generation rate, an increase in the

detoxification rate and upregulation of the repair process. It is assumed that CR has an impact on all these steps and therefore leads to an overall reduction of oxidative stress (3).

Adiponectin regulates several metabolic processes and has anti-inflammatory, anti-diabetic as well as anti-atherosclerotic effects (3,31). CR seems to be the most effective way to increase adiponectin levels in rodents (32), hence further evidence that CR increases lifespan (3).

There are many positive effects of CR, on the other hand, there is also one major risk factor – bone loss (3). Whereas CR could also protect rodents and non-human primates against sarcopenia (33,34), there is evidence that CR leads to bone loss and a decreased bone formation rate (35). However, other studies could not confirm this effect for example rats undergoing a lifelong restriction (36). Whether this side effect could also affect humans remain unclear (37), but people undergoing CR should be aware of this problem (3).

Further worth telling negative effects are reproduction and alveolar destruction (3,38). CR possibly increase the risk for emphysema and cause rapid destruction of alveoli (38). Female animals undergoing food restriction have reduced fertility but can increase reproductive capacity if switching back to *ad-libitum* (ad-lib) feeding (3,39,40). Supposedly the organism reacts to the deficiencies and wants to prevent becoming pregnant (3). However, these results rely on a 24h-48h fasting cycle (39) and not a typical CR. Actually, in other studies, CR even positively influenced the reproductive performance and mice and rats with CR had a greater performance than ad-lib animals do (3,41,42).

1.1.4 Human CR experiments

One of the most commonly known human experiments, Biosphere 2 (BIOS2) resulted in an unintentional CR of all participants. In September 1991 four men and four women moved into a closed ecological system to demonstrate the viability of human life in outer space. They were isolated via an airlock where no materials passed in or out for two years. Food was inside the closed space and all materials were recycled. Before isolation, 2500kcal per person were envisaged. Due to crop problems, food intake was limited, and the actual energy intake was 1780kcal. All Biospherians (the people inside the ecosystem) were in good health, non-smokers and followed a mainly vegetarian diet. They received mineral and vitamin supplements and had

access to water *ad libitum*. Inside the system every 2 weeks, medical tests were carried out by Walford (one of the Biospherians), who was a medical officer. After 6 months both sexes had a significant reduction in body weight as well as BMI and had a body fat composition in the lower region but were still healthy. Systolic as well as diastolic blood pressure were reduced. Serum cholesterol decreased in both genders whereas triglycerides were only decreased in men. Mean fasting glucose was lowered in both sexes. The conclusion of BIOS2 was, that this diet low in calories and fat had beneficial effects on health and that physiological changes in humans are similar to calorie-restricted rodents (17).

Towards the end of World War II, 36 healthy young men took part in Ancel Keys experiment at the University of Minnesota which resulted in a gruelling medical experiment. The aim was to see how civilians would be affected physiologically and psychologically by semistarvation and how they could be best rehabilitated after the dietary restriction. The diet started with 3 months of a standard diet containing 3200kcal followed by a semistarvation period with 1800kcal for 6 months and at the end 3 months of rehabilitation. Rehabilitation was separated into 4 groups with different energy intake, protein and vitamin levels. Keys remarked that 2000kcal/ day are not an appreciable rehabilitation, but if calories are abundant, extra protein and vitamins could help. A proper energy intake for rehabilitation would be 4000kcal. The semistarvation period aimed to reduce 1,1kg/week and a 25% weight reduction at the end of this period. With advanced semistarvation, participants complained about less energy, tiredness, hair loss, reduced coordination, and less tolerance to cold temperatures. They became irritable, impatient with each other and less motivated. Moreover, neurological deficits, the loss of sex drive, anaemia, and skin changes were further difficulties during the study. The rehabilitation was much more difficult than expected. Recovery lasted, depending on the participants, between 2 months and 2 years and tiredness and weakness improved slowly. All in all, the participants would do it all over again and believed that they did not have any negative long-term health effects (43). It is important to mention, that this diet was a diet during the war and far from an optimum diet.

In 1994 the CR Society was born, a non-profit organisation in the US. The major aim was to motivate a large population to learn about CR via a FAQ (frequently asked question) tool which still exists today in a better version. Additionally, the members had the opportunity to get in touch with other members and support each other in various ways. Today conferences are

planned, and newsletters inform about studies dealing with CR. Furthermore, society is working together with the media to educate the public (44).

1.1.5 CR and bile acids

Regarding fasting, the effect on the bile acid (BA) metabolism is mainly due to the regulation of the rate-limiting enzyme CYP7A1 (45,46). Insulin reacts to a restricted nutrient intake, as it decreases during fasting- and re-feeding cycles (47). It suppresses the transcription of CYP7A1, thus CYP7A1 expression increases during fasting (48,49). CR, similar to intermittent fasting, results in lower glucose and insulin levels. This is why CR can increase CYP7A1 expression and therefore BA concentrations (50). Additionally, Gregor et al. described, that CYP7A1 is upregulated during 25% CR in the liver (51). It has been shown that 40% CR increased BA pool size up to 162% since total BA concentration in the gall bladder and the small intestine increased. However, CR did not change BA levels in the liver. Normally BAs at high concentrations are considered cytotoxic but CR-induced higher BAs levels are not high enough to cause hepatotoxicity (50).

Moreover, there are differences between primary and secondary BAs as well as the conjugation status. In the SI (small intestine), CR led to an increase of primary, secondary, and conjugated BAs, however, unconjugated BAs in the SI were not influenced by CR. Furthermore, differences can be found between the individual BAs. When it comes to genes and transporters, which are regulating the BA metabolism, they correlate with the increase of BAs (50). For example, TGR5 is especially activated by LCA and DCA (52). Hence, an increase in DCA concentration may increase TGR5 activation. Additionally, an interesting finding revealed that CYP8B1 expression is decreased among CR (50). Another study suggests that the increased BA synthesis during fasting could be explained as a compensatory response to deficiencies in the enterohepatic circulation (EC) (53).

All in all, the effect of CR on BA metabolism has not been investigated satisfyingly yet. More information about BA transporters, receptors, and genes, involved in BA metabolism is needed.

Therefore, this master thesis focuses on the effect of CR on different BAs, conjugation of BAs, BA transporters, and genes connected to the BA metabolism.

1.2 Bile acid metabolism

BAs are the end products of the cholesterol metabolism and are generated through a cholesterol breakdown (54,55). In the liver, cholesterol gets converted into the primary BAs CA (cholic acid) and CDCA (chenodeoxycholic acid) via two pathways in humans. In mice, cholesterol gets converted into CA and muricholic acid (MCA) (53). Cholesterol gets first converted into 7α-hydroxycholesterol with the first, and rate-limiting, enzyme cholesterol 7α-hydroxylase (CYP7A1) which is regulated by a negative feedback system (55). The classical pathway consists of CYP7A1 and microsomal sterol 12α-hydroxylase (CYP8B1) and converts cholesterol into the primary BAs CA and CDCA, as mentioned before. CYP8B1 is required for the synthesis of CA, otherwise, only CDCA would be produced. The alternative way, also described as the acidic pathway, is initiated by sterol 27-hydroxylase (CYP27A1) and is responsible for about 9 %-10% of all BA synthesis in humans (54,56,57). In this pathway, BAs are mostly produced by oxysterols and cholesterol is less substrate for BA production (56). In mice, the acidic pathway accounts for about 25% of all BA synthesis (56,57). This pathway could be quantitatively important in neonates and patients with liver diseases (54). Afterwards, the primary BAs are conjugated to taurine or glycine prior to their secretion in bile (53). In humans, BAs are conjugated with a ratio of glycine to taurine 3:1 whereas in mice around 95% of the BAs are amidated to taurine. This amidation increases ionization and solubility at physiological pH, prevents calcium precipitation, minimizes passive absorption and is resistant to pancreas carboxypeptidases cleavage (54). Then BAs are immediately secreted into bile and collected in the gallbladder. If consuming food, the enterohormone cholecystokinin (CKK) stimulates the gallbladder and stimulates the release into the duodenum. BAs are important for the digestion and absorption of lipids and fat-soluble vitamins (58). About 95% of these BAs are absorbed in the ileum via the ileal bile acid transporter (=IBAT) and are transported back to the liver. This cycle is described as the EC. Only around 5% of BAs reach the intestine, where they are deconjugated by the microbial bile salt hydroxylase and converted to secondary BAs LCA (lithocholic acid) and DCA (deoxycholic acid) by gut bacteria (53,55). The 7α-

hydroxyl groups in CDCA can also be epimerized to ursodeoxycholic acid (UDCA) (54), which is a secondary BA in humans but a primary BA in rodents (53). After building secondary bile acids, these are conjugated to taurine or glycine again (Figure 1). Except, a small percentage of BAs, that are lost in faeces, are deconjugated (58).

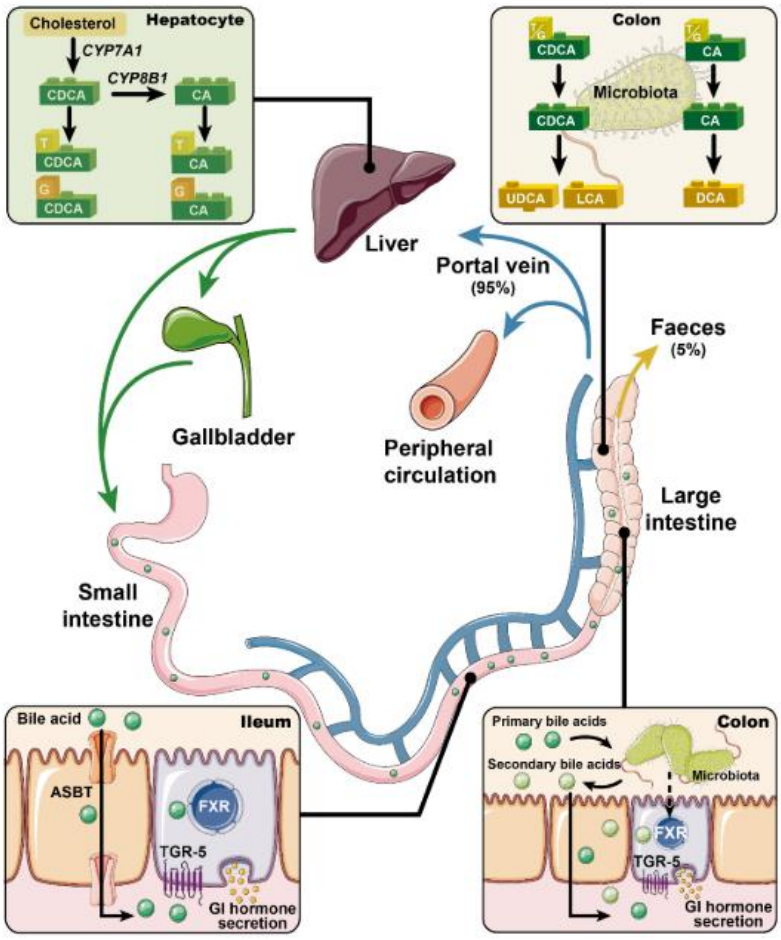

Figure 1: Bile acid metabolism, synthesis and enterohepatic circulation (53).

Every day 3g of BAs are cycling in the EC of humans about 4-12 times consisting of 40% CA and CDCA, 20% DCA and a trace amount of LCA. Approximately 0.5g BAs per day are lost with the faeces and spiked with *de novo* synthesis in the liver for maintaining a constant BA pool (54). The replenishing of the BA pool is regulated by FGF19 in humans and FGF15 in mice (53). In faeces, bile acids are deconjugated and mainly consist of DCA and LCA (58).

Findings underline the assumption that BA synthesis is regulated by a negative feedback control (54) which is regulated by the rate-limiting enzyme CYP7A1 (Figure1) (58). Rats fed with BAs

strongly reduced the enzyme activity, hence BA synthesis was downregulated (54). Thus, CYP7A1 controls BA production and defines the pool size (59). Furthermore, several factors could affect CYP7A1 such as inflammation, steroid hormones, and insulin. Again farnesoid X receptor (FXR) plays a critical role in the BA mechanism since it downregulates CYP7A1, CYP8B1 as well as CYP27A1 transcription (58). Additionally, the SHP (small heterodimer partner), an orphan nuclear receptor, is involved in the feedback mechanism, since it gets expressed by FXR. SHP then binds to LHR-1 and inhibits the promoter of CYP7A1 (60).

1.2.1 BAs regulating glucose and energy homeostasis

For a long time, BAs have been considered exclusively for their function as emulsifiers of fat and fat-soluble vitamins. Recently it has become clear that BAs bind to numerous receptors, especially FXR and TGR5 which is important as BAs act as pivotal signalling molecules, regulating the glucose-, lipid- and energy metabolism. A high turnover of BAs in the EC goes along with a high expression of FXR and TGR5 in the liver and intestine, whereupon gastrointestinal hormones are secreted. Postprandial BAs plasma concentrations correlate positively with the appetite and energy intake suppressing glucagon-like peptide-1(GLP-1) and peptide YY (PYY). Concerning FXR and TGR5 in this context, FXR signalling is still controversial but TGR5 stimulation on L-cells results in the secretion of both hormones. Moreover, TGR5 promotes intestinal motility as well as gallbladder relaxation and refilling. Concerning TGR5-mediated GLP-1 and PYY release, this effect depends on BA translocation from luminal to the basolateral side (53).

BAs are likely to affect energy balance through the secretion of gastrointestinal hormones (53). Supplementation of CA or UDCA has been shown to prevent weight gain in mice fed a high-fat diet possibly due to TRG5 related energy expenditure increase (61). Regarding humans, rectal administration of TCA suppressed hunger by supporting the secretion of GLP-1 and PYY (53).

Moreover, it has been shown, that insulin regulates CYP7A1 in rats. A lack of insulin results in the initiation of CYP7A1, whereas insulin represses the gene. During fasting, CYP7A1 is upregulated (54).

1.2.2 Immune regulatory effects of BAs

Bile acids are an important part of the immune system since BA receptors are expressed in several cells of the innate immune system. In the colon, as well as in the distal ileum, primary and secondary BAs activate TRG5 and FXR, which then regulates leukocyte trafficking and macrophage differentiation. Molecules like LPS activate macrophages by Toll-like receptors, hence the production of pro-inflammatory cytokines is induced. These cytokines, such as TNF-α, IL-6, and IL-1β are polarizing M0 macrophages to a pro-inflammatory phenotype which can produce effector T-cells. Bile acid activated receptors TRG5 and FXR reverse the inflammatory pathway to an anti-inflammatory pathway by polarizing macrophages to an anti-inflammatory phenotype. Besides, both receptor pathways are blunting NF-κB dependent gene expression (55).

1.2.3 BAs and their antimicrobial properties

BAs play an important role in bacterial overgrowth in the intestine and have antimicrobial effects (54,58). They are considered to influence gut microbiota by damaging bacteria cell membranes as BAs interact with membrane phospholipids (58,62). Especially DCA is able to affect the microbiota (58). Bacterial overgrowth, as well as mucosal injury, can be the result of an obstruction of the bile flow (54). BAs can reverse the obstructive effect, which is modulated by FXR-induced gene expression which is associated with an inhibition of bacteria damage (58).

1.2.4 Human diseases and BAs

Abnormal BA metabolism could be a birth defect due to mutations or enzymes, or transporter deficiencies. These abnormalities lead to altered steroid side chains which are toxic and could result in cholestatic liver disease in early childhood, up to neurological diseases at an advanced age. CYP7A1 and CYP7B1 deficiencies are one of the congenital disorders leading to abnormal BA metabolism which could be treated effectively with BA replacement therapy. Moreover, a cholestatic liver disease could be caused by a disruption of bile flow, which results in an accumulation of toxic BAs in the liver as well as increased BAs in the systemic circulation but

a lack of BAs in the intestine. Congenital cholestasis leads to pruritus, jaundice, and low absorption of fat-soluble vitamins which in turn leads to slow growth and progressive liver damage. Disruption of the bile flow could be caused by a blockage of the bile duct through gallstones, tumours or transporter defects (54).

Another application field of BAs regarding diseases is diabetes mellitus type 2 (T2D) and obesity (53). Concerning obesity, it has been shown that fasting plasma BA levels are increased due to an expanded BA synthesis. Augmented synthesis can be reflected by an expansion of 7α-hydroxy-4-cholesten-3-one (63–65). Additionally, production, as well as faecal excretion, of secondary BAs is increased which could be attributed to dysbiosis and therefore an impaired energy metabolism. Likewise, a shift of the BA composition was observed towards more 12α-hydroxylated bile acids which are more effective in emulsifying dietary fats, hence promoting fat digestion (53).

T2D patients have shown elevated levels of hepatic bile acid synthesis, especially CA, and an increased fasting BA concentration. Negatively associated with an increased risk for diabetes are unconjugated primary and secondary BAs whereas conjugated BAs, primary and secondary, are positively associated. Hence, CA, DCDA, and DCA show a decreased T2D risk, and TCA, TDCA, and TUDCA show an increased risk. These findings suggest that intestinal reabsorption of conjugated BAs, as well as impaired catalysis of conjugated BAs by bile acid CoA-Amino acid N-acetyltransferase (BAAT), could contribute to the development of T2D and additionally, the BA profile may be a useful tool for predicting obesity and T2D (53).

1.3 Receptors and Transporter involved in the BA metabolism

1.3.1 FXR, farnesoid X-receptor

FXR plays a key role in the BA metabolism. It is involved in the regulation, biliary secretion, intestinal absorption, and hepatic uptake of bile acids. FXR is capable of inhibiting transactivators of CYP7A1 and Cyp8B1 and regulates BA conjugation by bile acid CoA synthase (BCAT) and BAAT gene transcription (54). BCAT and BAAT are the 2 main enzymes conjugating BAs (58). Moreover, FXR activates a bile salt export pump called ABCB11, which is in charge of utilizing ATP hydrolysis to excrete conjugated BAs against a biliary BA concentration. Additionally, FXR induces the ABCG5 expression which is responsible for

biliary cholesterol excretion (54). Inside the enterocytes BAs, are binding to the BA-binding protein, which is highly expressed by FXR (54,66). When it comes to absorption into portal circulation Ostα and Ostβ dimers act as BA efflux transporter (54). Again, FXR is a mediator as it induces Ostα/β gene transcription (67). Summarizing FXR plays an important role in the BA metabolism as it is involved in the synthesis, secretion, reabsorption, and uptake into the hepatocytes. Defective regulation of the FXR gene leads to impaired EC of BAs. Hence, defects of FXR result in cholestatic liver disease. Pregnancy x-receptor (PXR) and FXR are also discussed as detoxification mediators of BAs and therefore have an important impact on cholestasis (54).

In mice, CA has been identified as the most potent FXR ligand whereas two primary BAs αMCA and βMCA, derived from CDCA, are FXR antagonists. In humans, CDCA is the most efficient FXR ligand (55).

The intestinal microbiota is regulating FXR signalling which is observed at the conversion from primary into secondary BAs and by regulating the synthesis. However, FXR signalling through the bacteria, could only be described for the ileum and not in the liver. Another interesting finding is the BA pool size which is smaller if gut bacteria is present (68).

1.3.2 Takeda G-protein receptor 5 (TGR5)

TGR5, also known as G-protein bile acid receptor 1 (GPBAR1), is a cell-membrane receptor for bile acids (55). It is expressed in many tissues such as gall bladder, liver, spleen, intestine, kidney, adipocytes, skeletal muscle, and macrophages (54). Moreover, TGR5 has been detected in the Kupffer cells, however, not in hepatocytes (54,58). There are differences between the binding affinity of BAs and TRG5. TLCA has the highest affinity, followed by LCA, whereas CA has the lowest affinity to TRG5 (54).

TGR5 induces gallbladder refilling and relaxation, promotes intestine motility and has a stimulatory effect on GLP-1 (53). Moreover, the activation in the ileum results in increased PYY levels, thus producing an anorexigenic effect. In macrophages and Kupffer cells the activation leads to an inhibition of the LPS-induced cytokine production (58) which helps to prevent atherosclerosis. Besides the inhibited cytokine production of macrophages, TGR5 in

the liver and intestine inhibits NF-kB-mediated inflammatory cytokine production. Therefore, TGR5 signalling through BA activation may play an important role in the protection against inflammatory diseases like fatty liver disease, atherosclerosis, IBD, or diabetes (54).

TGR5 knockout mice have shown a decreased BA pool and accumulation of fat if fed with a high-fat diet (54). Once more, these findings make it clear how important TGR5 in the BA metabolism is.

1.3.3 Ileal apical sodium dependent bile acid cotransporter (IBAT)

The ileal apical sodium-depended bile acid cotransporter (IBAT, also known as ASBT) is a glycoprotein which belongs to the SLC10 family and is characterised by having important functions as bile acid transporters. IBAT is located in the apical brush border membrane of the ileal enterocytes and coordinates the initial uptake of bile acids. As the name already suggests, the highest mRNA or protein expression was shown in the ileum rather than in the proximal intestine or colon. IBAT is expressed at those tissue sites, which implements the EC of BAs. The highest activity or expression was found in the villus but not in the crypt enterocytes. Additionally, IBAT is expressed in renal proximal tubule cells, in the biliary epithelium and the kidney. In kidney and ileum, the majority of BAs available in the adjacent lumen get absorbed. The physiological substrates of IBAT contain the major unconjugated BAs CA, DCA, CDCA and UDCA, but also their taurine conjugates. Since IBAT plays an important role in EC, dysfunction or defects in the regulation of IBAT may be interesting for the pathogenesis of gastrointestinal disorders (69).

1.3.4 Organic Solute Transporter subunit alpha/beta (Ostα/β)

Ostα/β is a BA transporter, which is responsible for the BA export across the basolateral membrane (69). It seems to be the major efflux transporter for BAs in the intestine since it is responsible for the excretion of BAs into the portal circulation (54). It is located on the lateral and basolateral plasma membrane of the ileal enterocyte and is highly expressed in the hepatocytes and also in cholangiocytes and renal proximal tubule cells (69,70). Moreover, organic solute transport plays an important role in the efflux of secondary BAs in the sinusoidal

membrane (54). Positive expression regulation of the organic solute transporter is reliant on the activation of FXR by BAs (69). One of the major differences between Ostα/β and the ileal BA transporter (IBAT) are the substrates they have been identified to. Whereas for IBAT only BA substrates have been identified, for Ostα/β also non-BA substrates were found (70) such as estrone-3-sulfate, prostaglandin E2, and digoxin. In humans, higher gene expression levels were found in the liver whereas in rodent liver Ostα/β expression was almost undetectable. In case of cholestasis in both, humans and rodents, the gene expression of Ostα/β raised dramatically. If the Ostα gene is inactivated, BA absorption will be impaired and BA metabolism is altered. Furthermore, experiments with Ostα null mice have shown, that the efflux of BAs is impaired, and BAs accumulate in the ileal enterocytes. As a result, FXR activates a greater secretion of FGF15 which paradoxically downregulates BA synthesis (69).

1.3.5 Fibroblast growth factor 15 (FGF15)

FGF15 mouse ortholog to human FGF19 works as an enterohepatic signal and activates hepatic FGFR4 (FGF receptor) which signals the inhibition of CYP7A1 expression. This mechanism is FXR dependent (54). In detail, FXR induces FGF15 that activates a pathway depending on a c-Jun N-terminal kinase (JNK). Mice, which are lacking FGFR4, show an increased BA pool size since the JNK activity is reduced and the expression of CYP7A1 is intensified. Moreover, SHP is involved in the repression of BA synthesis (60). Interestingly, this effect was only detectable concerning the intestinal FXR. This indicates that BA inhibition of CYP7A1 transcription requires intestinal FXR. Moreover, BAs do not induce FGF15 in the liver, therefore the feedback pathway still stays unclear (54).

1.3.6 Fibroblast growth factor 21 (FGF21)

The fibroblast growth factor 21, together with the FGF15 and FGF23, belongs to the atypical fibroblast growth factors since they lack the conventional heparin binding domain. Hence, FGF21 could act as a hormone and diffuses away from the original tissue (71). FGF21 is mainly expressed in the liver, where it induces an endo-paracrine response to prolonged fasting and gets upregulated by PPARα (72). FGF21 induces gluconeogenesis, fatty acid oxidation and

ketogenesis which are all characteristic of fasting. Since it is only responsible for gluconeogenesis and not for glycolysis, it is assumed that FGF21 only plays an important role in prolonged fasting and not in the early stages. Moreover, the fibroblast growth factor is able to put the mice in a hibernation-like state of torpor and plays a role in reducing the growth-hormone-signalling pathway since it reduced the circulating IGF-1 (71). In humans, however, FGF21 does not regulate an immediate starvation response and additionally, blood levels rise just after 7-10 days of fasting. Nevertheless, FGF21 plays an important role in fasting and starvation (72).

Besides the fasting aspect, FGF21 is additionally important for the regulation of sweet-taste behaviour as well as for regulating a high-fat diet (71,73). FGF21 knockout mice had increased consumption of high-sucrose, glucose, and fructose diets whereas mice with FGF21 overexpression preferred the chow diet. It is assumed that FGF21 plays a role in a liver-brain hormonal axis with a feedback loop which regulates carbohydrate intake (73). Concerning a high-fat diet, FGF21 overexpression in transgenic mice resulted in resistant weight gaining, reduced plasma TG, and improved insulin sensitivity as well as glucose clearance (71).

1.3.7 Pregnancy x-receptor (PXR)

PXR, a nuclear receptor, is characterised by supporting the body's xenobiotic defence system (74). It gets activated by BAs, sterols and especially clinical drugs and can induce the drug-metabolizing enzymes CYP3A, CYP2B and CYP2C. Especially, CYP3A4, the most important cytochrome P450 enzyme, is in charge of metabolizing more than 50% of clinical drug (75). Additionally, CYP3A4 plays an important role in hydroxylating BAs as well as MRP2 and OATP2, which are transporting BAs across the hepatic canicular and sinusoidal membranes. Besides CYP3A4, PXR is involved in many genes regulating the BA metabolism. CYP7A1, the rate-limiting enzyme, is regulated by PXR. Studies have shown, that PXR null-mice demonstrate dysregulated CYP7A1 levels in both, basal expression as well as repression (74).

LCA (lithocholic acid), especially the taurine-conjugated version TLCA is described as a toxic BA since it could cause cholestasis. Due to the impairment or cessation of the bile flow, as well as accumulation of BAs in liver or serum, cholestasis can occur. This could result in total liver failure (74,76). This toxic BA in pathophysiological levels is activating both, human and

rodent PXR which is then involved in the detoxification and removal of this BA. PXR boosts the expression of a programme of genes, which are part of detoxification (74). Again, FXR plays an important role in the BA metabolism as it cooperates with PXR in the removal of toxic LCA levels. Moreover, conjugation and hydroxylation debilitate the potentially harmful effects of LCA (77). These findings indicate that PXR is an LCA sensor and prevents LCA toxicity in the liver (78). Furthermore, UDCA can support the CYP3A4 expression and activate PXR which again underlies the anticholestatic effect of PXR (74).

It can be taken for granted, that besides BAs, also oxysterols and cholesterol are able to activate PXR. PXR null mice, consuming a high cholesterol diet, developed hepatorenal failure which may indicate that PXR is important for the detoxification of cholesterol metabolites. Furthermore, this supports the possible role of PXR activating CYP27A1 (the alternative BA pathway) as an adaptive response to remove excessive cholesterol in the intestine (78).

1.3.8 Liver X receptor alpha and beta (LXR α/β) and the ATP binding cassette transporters ABCA1 and ABCG1

LXR α/β belong to the ligand activated transcriptional factors and their key role is controlling lipid metabolism and inflammation. Besides the lipid metabolism, it has been shown, that LXR α/β play an important role in the BA metabolism as well as in cholesterol and triglyceride metabolism. LXR protects cells from cholesterol overload through two different elimination ways in the liver. First, it promotes CYP7A1 expression, second, it promotes the mRNA expression of ABCA1 and ABCG1. ABCG1 and ACBA1 are the so-called ATP-binding cassette transporters and play an important role in transporting cholesterol from hepatocytes to bile canaliculi. Besides the cholesterol overload protection in hepatocytes, LXR could additionally promote reverse cholesterol transport in macrophages. Therefore, LXR induces the expression of the ATP-binding cassette transporters, which support the efflux to high-density lipoprotein (79). These transporters synergistically stimulate the cholesterol efflux in macrophages and are likely to be expressed in the basolateral membrane of the enterocytes (78).

Apart from the classical BA pathway, LXR also plays a role in the acidic pathway. If the intestine is exposed to high levels of cholesterol, CYP27A1 may produce oxysterols which

further activate LXRα for regulating the efflux of cholesterol. Furthermore, PXR may stimulate CYP27A1 due to high cholesterol levels in the intestine (78).

Moreover, LXR is important for increasing the synthesis of fatty acids by upregulating the sterol regulatory binding protein (SREBP1c). LXR also regulates glucose homeostasis and had made its name as an antiatherogenic, antidiabetic, and immunomodulatory receptor (79).

1.3.9 Sterol regulatory element binding protein 1c (SREBP1c)

SREBPs are basic helix-loop-helix leucine zipper transcription factors (TFs) which control genes responsible for cholesterol biosynthesis and receptor mediated uptake of LDL. SREBP-1 especially is involved in lipogenesis and fatty acid synthesis (80). SREBP1c is the most relevant isoform in the adult liver. Particularly in rodents, its expression is regulated by insulin, cholesterol derivates, triiodothyronine and other endogenous molecules (81). It has been shown that an insulin treatment increased SREBP1c expression considerably (82) and it is managed by the nutritional status. Fasting results in lower mRNA levels whereas feeding strongly induced SREBP1c mRNA levels (81). Under low cholesterol conditions it, will probably not be induced (82). Furthermore, SREBP1c is important for the expression of glycolytic and lipogenic genes (82). Irregularities of the TF may be a key to linking insulin resistance with other metabolic disorders that are associated with obesity (81).

It has been shown, that LXRα/β regulates SREBP1c. The activation of LXR induces SREBP1c, which results in a rise in VLDL (80,83). Additionally, PPARα plays an important role in lipogenesis. Overexpression inhibits SREBP1c promoter activity due to competing with the LXR/RXR heterodimer. Hence, lipid homeostasis is a cross-talk complex between the three TFs SREBP, LXR, and PPARα (80).

Concerning BAs, it has long been known that triglyceride (TG)homeostasis is affected by BAs and that there is a reciprocal relationship between BA synthesis and TG production (80). On the one hand, CDCA can reduce hypertriglyceridemia as a treatment for cholesterol gallstone (84–86), whereas BA-binding resins in humans induce the production of VLDL (80,85). Two mechanisms are explaining this relationship between BAs and TGs. First, BAs are ligands for FXR, which activates several genes involved in TG levels such as SHP, PPARα, and ApoC-II

(Figure 2). SHP interferes with SREBP1c expression due to its inhibiting effect on LXR (Figure 2). On the other hand, decreased BA synthesis could increase cholesterol and oxysterol levels, thus SREBP1c is activated which could result in decreased TG production (Figure 2). Cholic acid is underlying the TG lowering effect of BAs since it lowers serum as well as hepatic TG levels. Moreover, it decreases SPREBP1c and other lipogenic genes. In addition, CDCA increases the SHP expression which makes it clear that BAs significantly affect the expression of SREBP1c (Figure 2) (80).

Nevertheless, it is important to mention that human SREBP1c promoters have 42% similarity to mice promoters, hence different pathways and mechanisms may be regulating SREBP1c in humans and mice (81). Mice with CYP7A1 deficiency do not develop hypertriglyceridemia whereas humans do. Furthermore, resins, which are used for the management of human lipid disorders, could result in the negative side effect of higher TG levels. Summarizing, SREBP1c is the initial major target of BAs to lower TG levels (80).

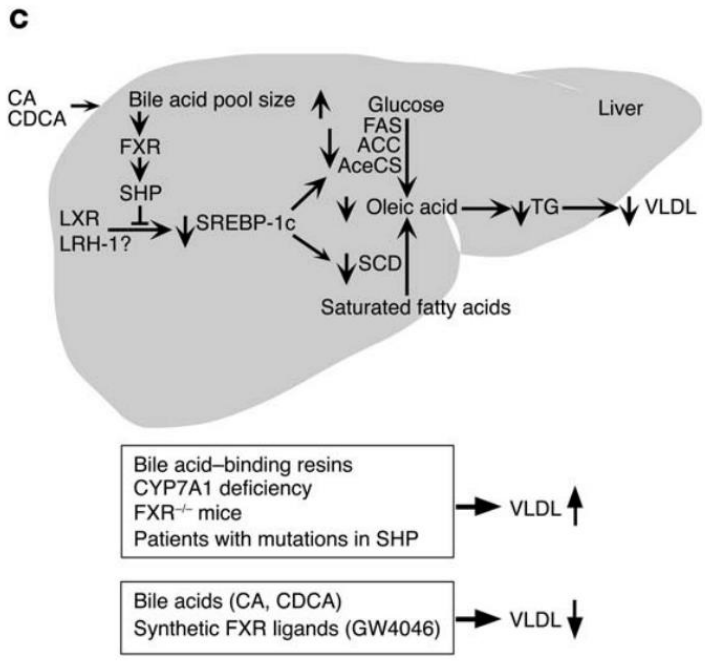

Figure 2: Bile acids downregulate SREBP1c which results in lowered TG and VLDL levels (80).

1.3.10 (CYP8B1) 12 α-hydroxylase

CYP8B1 is a hepatic microsomal hydroxylase and belongs to the cytochrome P450 family. As already mentioned, it is involved in the BA synthesis as it regulates the ratio of CA over CDCA which is secreted in bile (87). Additionally, Chiang et al. described the function of CYP8B1, as it is required for the synthesis of CA. If CYP8B1 is missing in an organism, only CDCA is formed (54). Moreover, the ratio between CA and CDCA, which is controlled by CYP8B1, is important for controlling the solubility of cholesterol (87). An increase in CA may results in a higher solubility of cholesterol in bile, thus increasing cholesterol absorption. Cholesterol feeding decreases the enzyme activity of CYP8B1 in mice, whereas CYP7A1 activity is increased (88). Interestingly, it depends on the type of fasting, whether CYP8B1 mRNA expression is increased or reduced. Fasting stimulated mRNA expression, whereas restricted-feeding reduced expression. Feeding inhibits activity as well (89).

Moreover, there is a huge difference between cholesterol homeostasis in rats and humans. In rats, cholesterol absorption depends on CA, which means that cholesterol absorption is dependent on CYP8B1. Decreasing CYP8B1 will decrease cholesterol uptake in the intestine, hence rats consuming a high cholesterol diet will reduce cholesterol absorption due to the downregulation of CYP8B1 which facilitates cholesterol homeostasis. This mechanism is lacking or less efficient in humans (88).

Furthermore, mice lacking CYP8B1 are protected against hypercholesteremia and gall stones due to the altered BA pool. Interestingly, because of the lack of CYP8B1, GLP-1 levels are increased, which results in improved insulin secretion and glucose tolerance. Since CA is missing, more free fatty acids are reaching the L cells, which correlates with an increased GLP-1 secretion. Thus, CYP8B1 lacking, could be a useful treatment for the management of diabetes as well as beneficial for the metabolic syndrome (90). Besides, a further paper described that the regulation of CYP8B1 is related to obesity-related inflammation, non-alcoholic fatty liver disease and diabetes. Both, BA pool composition and pool size are crucial for the regulation of hepatic lipid metabolism as well as metabolic disease (89).

1.4 Differences in the BA metabolism between mouse and human

As seen in other animal studies, the question will always remain if the results are transferable from animals to humans. Related to this master thesis, differences between BA metabolism, differences in the composition of BAs as well as differences in BA synthesis would be interesting to compare.

Regarding the BA composition, there are some major differences between mice and humans. The most abundant BA in plasma in mice is the muricholic acid (MCA) (91), which is a 6-hydroxylated hydrophilic BA (92), whereas in humans the most present BA is DCA. Moreover, the CA/CDCA ratio is high in mice, and in humans, however, this ratio is very low (91). As already mentioned before, the amidation of BAs in mice is mostly done by binding taurine, whereas in humans most BAs are glycine amidated. Additionally, there are also differences between the species, relating to the number of hydroxyl groups. In mice tri-OH BAs are dominating, in humans, on the contrary, di-OH BAs are more dominant (91). UDCA, a secondary BA in humans, is a primary BA in mice (53).

A further difference to mention is the toxicity of BAs, which strongly correlates with the hydrophobicity of BAs. Hydrophobic BAs are more cytotoxic than hydrophilic BAs. LCA and DCA are hydrophobic, therefore they are more toxic than CA, UDCA and MCA, the hydrophilic BAs. This leads to the conclusion, that mice's BA profile is less toxic than humans' since mice consist mainly of hydrophilic BAs (91).

Taking a closer look at the BA metabolism, the basal turnover of BAs and cholesterol and the circulating LDL is much higher in mice. The LDL-cholesterol concentration, however, is much lower, compared to humans. On the other hand, an interruption of the EC tends to evoke stronger effects on cholesterol and LDL levels in humans. Since taurine-conjugated MCA has antagonistic FXR properties, this may explain the differences in the BA metabolism between the two different species (92). When it comes to BAs acting as ligands for FXR, CA is the most potent ligand in mice. In contrast, CDCA has the best potential as an FXR ligand in humans (55).

Another important difference between these two species concerns the effect of cholestasis and BA synthesis. In mice BA production is upregulated, in humans conversely, it is suppressed if the bile duct is ligated (92).

In addition, a human-like phenotype knockout mouse (KO) called Cyp2c70 was created, to compare the wild type with the KO human-like mouse. Since the gene Cyp2c70 is responsible for MCA production, disruption of the gene resulted in the elimination of the mouse dominant BA, thus a more human-like phenotype was established. It is expected that this KO mouse could be a useful tool concerning human diseases for future research (92).

Table 1: Overview of the main differences regarding BA metabolism between mice and humans (53,55,56,69,91,92)

	
Predominantly amidated to taurine	Predominantly amidated to glycine
High CA/CDCA ratio	Low CA/CDCA ratio
Tri-OH BAs dominating	Di-OH BAs dominating
More hydrophilic BAs – less toxicity	More hydrophobic BAs – more toxicity
UDCA – primary BA	UDCA – secondary BA
Higher synthesis of cholesterol and BAs	Lower synthesis of cholesterol and BAs
Faster clearance	
Lower levels of LDL-cholesterol	Higher LDL-cholesterol levels
Production of MCA	No MCA
Dominate BA: MCA	Dominant BA: DCA
Acidic pathway makes up ~25%	Acidic pathway makes up ~ 10%
CDCA's most potent FXR ligand	CA's most potent FXR ligand
	Disruption of EC leads to bigger impact on cholesterol and LDL metabolism

1.5 Intermittent fasting (IF)

Intermittent fasting, shortly IF, is distinguished by a period of voluntary abstinence from food and drinks in humans. It was already practised in ancient times and raised in a variety of different formats. Alternate day fasting, time-restricted fasting, modified fasting regimes and religious fasting such as Ramadan are some of the most popular IF types. About "Alternative day fasting" a lot of beneficial health aspects were described. On fasting days no calories are consumed and on feeding days drinks and foods are consumed ad-lib. It has been shown that alternative day fasting is as effective as CR concerning body weight loss, fasting- glucose and insulin concentrations. In obese mice, it reduced TG, total plasma cholesterol, liver steatosis and inflammatory gene expression and had a positive impact on cancer risk factors (93). In addition, in rodents, IF protects against diabetes, cancers, heart disease, and neurodegeneration as described in Longo et al. (94).

1.6 Fasting mimicking diet (FMD)

As the name of the diet suggests, it mimics the effects of fasting which relate to markers of stress reduction. This means low glucose and IGF-1 levels and high levels of ketone bodies and IGFBP-1. FMD is characterised by fasting phases which are followed by an ad-lib phase. During fasting, fewer calories and a low protein diet are responsible for the effects similar to fasting (95). Concerning humans, FMD is characterized by vegetable-based soups, energy drinks, energy bars, tea, snacks and supplements, which ensure the supply of vitamins, minerals and essential fatty acids (96). A four-day intervention followed by an ad-lib standard diet can promote health- and lifespan in mice. It has been shown that this fasting cycle has a clear effect on visceral fat, glucose, and IGF-1 levels in mice, however, this effect was reversed when mice turned back to the ad-lib phase of the cycle. Moreover, an FMD diet protects against inflammation which can play a key role in the development of age-related diseases such as cancer. In older mice, FMD leads to improved cognitive performance and hippocampal neurogenesis. Bone mineral density loss, rejuvenation of the immune system and reduced cancer incidence are just some of the positive effects of FMD (95). Regarding diabetes type 2, Cheng et al. described that 4-days of a fasting-mimicking diet could have similar effects on metabolic changes to prolonged fasting. This means low levels of glucose and insulin on the

one hand and higher levels of ketone bodies and IGFBP-1 on the other hand. In the mouse model, FMD obtained glucose homeostasis and restored insulin secretion in type 1 diabetes (T1D) as well as in type 2 diabetes (T2D). Whereas in humans FMD reduced protein kinase A (PKA) and mTOR activity and induced Sox2 and Ngn3 expression and insulin secretion in T1D. Consequently, FMD in mice could restore the insulin production in pancreatic cells for both diabetes types whereas in humans only in T1D patients (96).

1.7 Ketogenic diet (KD)

The ketogenic diet (KD), which originated in 1921, is a diet applied for epilepsy treatment. It is characterised by a limited carbohydrate intake of foods with low glycaemic response and energy is mainly obtained from fat. The hallmark of a KD is a high level of ketone bodies, which are used for anaerobic energy production instead of glucose. Once glycogen stores are depleted, the body needs alternative energy sources via ketogenesis. Therefore, free fatty acids are converted into the ketone bodies acetone, acetoacetate, and ß-hydroxybutyrate and replace glucose as the main energy source. Ketone bodies are producing more ATP than glucose, which guarantees efficient maintenance of energy production even during CR (97).

Over time, four main types of KD have been established. The classical ketogenic diet (CKD), the medium chain triglyceride ketogenic diet (MCT KD), the modified Atkins diet (MAD) and the low glycaemic index treatment (LGIT) (98). CKD is described as a more restrictive diet to achieve a specific fat to carbohydrate ratio, which is traditionally 4:1. The main energy source is LCT (long chain triglyceride). MCT KD, on the other hand, consists mainly of MCT oil, about 60% are caprylic acid (C8) whereas 40% are capric acid (C10) (97). It was first induced by Dr. Huttenlocher and demonstrated the advantage that MCTs are absorbed more efficiently than LCT. Furthermore, MCT does not require carnitine for the transport into cell mitochondria for oxidation and can cross the blood-brain barrier and influence brain metabolism directly (97,99,100). A further advantage, less total fat is required since MCT oils deliver higher levels of ketosis than LCTs (97). Concerning MAD, this form is known as the less restrictive and more palatable diet compared to CKD. Thus, it goes along with greater compliance by older children, teenagers and adults (97). Additionally, LGIT is described as a less restrictive diet but

still more prescriptive than MAD (97,101). LGIT aims to stabilize blood glucose levels and prevent postprandial insulin fluctuations (102,103).

Concerning BAs, a high-fat diet leads to increased excretion of secondary BAs which are additionally a risk factor for colorectal cancer (58). This can be supported by Yoshitsugu et al., where a high-fat diet leads to higher excretion of secondary BAs as well. Furthermore, the result of this study was that TCA was the major BA in the bile and intestine consuming a high-fat diet. TCA possesses high water solubility and is available in sufficient quantities to form emulsions and guarantee lipid digestion. Therefore, TCA might increase as a response to high levels of fat in the diet (104). A high-fat diet is not the same as KD, but KD contains a high amount of fat. Moreover, a ketogenic diet in colitis mice has been shown to influence bile secretion pathway. TDCA and CA decreased in colon of colitis mice. Biosynthesis of primary BAs was downregulated whereas BA secretion was upregulated if a KD was applied (105).

1.8 Aims and questions

This master thesis aims to find out how CR influences taurine-conjugated and unconjugated BAs in mice. Moreover, to understand the whole mechanism behind BA metabolism, several transporters, receptors and transcriptional factors were measured to see the effects of several diets on BA metabolism.

The ambition was to find answers to the following questions:

Does fibre-rich bedding affect the BAs and if so, how? Does coprophagy have any influence? How are different types of bedding affecting BAs and BA excretion?

What is the role of the microbiota when it comes to BAs? How does faecal transplant (FT) from a CR mouse to an ad-lib mouse affect BA levels? Which role do antibiotics play, regarding the BAs and the microbiome? Does CR itself influence BAs or is the microbiome responsible for it?

Are other forms of restrictive diets such as IF, FMD and KD influencing the BA metabolism?

Are there any differences in the BA composition if taurine is supplemented with the diet? How does a higher amount of taurine differ from low taurine or no taurine diet in terms of the impact on BAs?

2 Methods

2.1 Material and equipment

2.1.1 LCMS Material

LCMS Shimadzu 8040:

o Autosampler SIL-20AC HT

o Liquid Chromatograph LC-20 AD

o Degassing Unit DGU-20A5

o Kinetex® 5 µm EVO C18 100 Å LC Column 150 x 3.0 mm

o LabSolutions (Software)

o High Pressure Switching Valve FCV-20AH2o UV/VIS Detector SPD-20A

o Column Oven CTO-20AC

HPLC vials: 2mL; Sigma-Aldrich Chemie GmbH

Precellys tubes: Fisherbrand™ Free-Standing Microcentrifuge Tubes with Screw Caps; FisherScientfic

2.1.2 qPCR Material

- **Dispenser:** 0,5 – 10µL, 5-100µl, Eppendorf Xplorer®; Eppendorf
- **qPCR plate**: Frame Star® 384 Well Skirted PCR Plate, Roche Style; 4titude® via Brooks Life Sciences
- **qPCR seal**: 4titude® via Brooks Life Sciences
- **Nano drop**: NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer; ThermoFisher Scientific
- **Block heater** (for cDNA):

- Block Heater, 2 Block, Digital, SBH130D, SBH200D; Stuart® via BioCote
- Liebisch Labtherm 2009 Digital Block Heater
- Thermostat 5320 Dry Bath Heat Block; Eppendorf; Netheler-Hinz GmbH; Hamburg

- **qPCR**: applied biosystems®, Quant studio™ 6 Flex Real-time PCR System, contains the OptiFlex™ Optics System; Life Technologies Holding (Singapore)
- Disposable cannulas: "Sterican® Gr. 14, G 23 x 1 1/4"" / ø 0,60 x 30 mm; Braun
- RNA Kit: peqGOLD HP RNA Kit; peQlab; VWR International GmbH (Erlangen)

2.1.3 General usage

- **Microcentrifuge tubes**: 1.5 mL and 2 mL; Eppendorf Austria GmbH (Vienna, Austria)
- **Pipette**: 2,5,10,100,200 1000 µl; Eppendorf Reference® 2; Eppendorf
- **Tips**: 0,1 – 20 µL, 10-100µ, 100-1000µl, epT.I.P.S.® 384; Eppendorf

2.1.4 BAs

- **Bile Salts**; MDL: MFCD00130648; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Chenodeoxycholic acid** (CDCA) ≥96 % ($C_{24}H_{40}O_4$); CAS: 474-25-9; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Cholic acid**, sodium salt (CA) ($C_{24}H_{39}O_5Na$); CAS: 361-09-1; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Deoxycholic acid**, sodium salt (DCA) ($C_{24}H_{39}NaO_4$); CAS: 302-95-4; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)

- **Dehydrocholic acid** (DHCA) ≥99 % ($C_{24}H_{34}O_5$); CAS: 81-23-2; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Lithocholic acid** (LCA) ≥95 % ($C_{24}H_{40}O_3$); CAS: 434-13-9; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Sodium Taurolithocholate** (TLCA) ($C_{26}H_{44}NO_5SNa$); CAS: 6042-32-6; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Taurocholic acid**, sodium salt (TCA) ($C_{26}H_{44}NO_7SNa$); CAS: 145-42-6; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Taurodeoxycholic acid**, sodium salt (TDCA) ($C_{26}H_{44}NO_6SNa$); CAS: 1180-95-6; Merck KGaA (Darmstadt, Germany)
- **Tauroursodeoxycholic acid**, sodium salt (TUDCA) ($C_{26}H_{44}NNaO_6S$); Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Ursodeoxycholic acid** (UDCA) ≥99 % ($C_{24}H_{40}O_4$); CAS: 128-13-2; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)

2.1.5 Bedding

- Arbocel Performance Small, pellets, cellulose; J. Rettenmaier & Söhne GmbH + Co KG (Vienna, Austria)
- Lignocel select, poplar, cubic; J. Rettenmaier & Söhne GmbH + Co KG (Vienna, Austria)
- RehoFix MK 3500, maize grains; J. Rettenmaier & Söhne GmbH + Co KG (Vienna, Austria)

2.1.6 Diets

- V1535 R/M-H Extrudate, autoclavable; ssniff Spezialdiäten GmbH (Soest Germany)

- LDT w/o Met, Cys; + 0,0% Taurine, 10% Casein; 10mm; ssniff Spezialdiäten GmbH (Soest Germany)
- NTD w/o Met, Cys; + 0,5% Taurine; 10% Casein; 10mm; ssniff Spezialdiäten GmbH (Soest Germany)
- LF 5% fat High Fibre 20% Cellulose; ssniff Spezialdiäten GmbH (Soest Germany)
- LF 5% fat High fibre 20% ferm. Fibre; ssniff Spezialdiäten GmbH (Soest Germany)
- FMD diet consisting of:
    - Broth mix, ABCO Laboratories, Fairfield, CA
    - EVOO, Mizkan American, Inc, Mount Prospect, Illinois
    - Essential fatty acid, Flora Incorporated, Lynden, Washington
    - Vegetable mix, The Synergy Company, Moab, Utah and Nature Foods, West Palm Beach, FL
    - Glycerol, Sigma-Aldrich, St. Louis, Missouri
    - Hydrogel, Clear H2O, Westbrook, Maine.
    - Fibre, Cellulose, #3425, Bio-Serv, Flemington, New Jersey
    - Mineral (AIN-93G-MX), Teklad, TD.94046, ENVIGO
    - Vitamin (AIN-93-VX), Teklad, TD.94047, ENVIGO
- Keto diets: ssniff Spezialdiäten GmbH (Soest Germany)

2.1.7 Further Equipment

- **Vortexer**: Reax 2000; Heidolph
- **Scale:** Sartorius Laborwaage Entris Model ENTRIS224-1S
- **Centrifuge big**: Thermo Scientific™ Megafuge™ 8; fisher scientific
- **Centrifuge small**: Microcentrifuges, ventilated/refrigerated, Micro Star 17 / 17R, VWR
- **Concentrator**: Concentrator plus/Vacufuge® plus, Eppendorf

- **Rocking shaker:** Fisherbrand™ Sea Star orbital shaker package; fisher scientific
- **Hot water bath tub**: GFL 1002; Müller-Scherr; Linz
- **Precellys shaker**: Homogenisator, Precellys® 24; VWR
- **Antibiotics mixture**: Sigma-Aldrich (St. Louis, MO, USA)

2.1.8 Reagents

- **Ethanol**: absolut EMPLURA®; Merck
- **Methanol**: ROTISOLV® HPLC Gradient Grade; ROTH
- **Acetonitrile**: ROTISOLV® HPLC Gradient Grade; ROTH
- **Formic acid**: ROTIPURAN® ≥98 %, p.a., ACS; ROTH
- **HPLC water**: ROTISOLV® HPLC Gradient Grade, ROTH
- **Ammoniumacetate:** ≥97 %, p.a., ACS, ROTH

2.2 Laboratory mouse C57BL/6

For the diet experiments male C57BL/6NRj mice were used and purchased from Janvier Inc. Labs (Le Genest, France). The mice were divided according to their respective diet group. The treatment started when they were 10 weeks old and lasted 2 weeks. Afterwards, the mice were dissected after euthanasia by isoflurane overdose. The mice were kept under a 12h light/12h dark cycle in a standard housing cage. Before the treatment, they were fed with standard chow.

The C57BL/6 mouse is a widely used laboratory mouse model and finds usage in many different experimental fields. The most common usage for this mouse model is immunology and antitumor activity studies, but it is also used for obesity studies and cellular signal transduction studies. Moreover, the type is used for nutritional studies such as lipid metabolism and glucose metabolism (106).

2.3 Experiments

In this thesis, experiments with different diets, bedding and different supplementations were carried out. In each experiment, the groups contained 8-10 mice. For the control group, a standard chow from ssniff Spezialdiäten GmbH was used. Mice tend to eat around 4g of food per day if kept ad-lib. The standard chow contains a mix of grains and grain by-products, oil seed products, minerals, vegetable oils, vitamins and trace elements.

2.3.1 Experiment I: Bedding influence

Figure 3: Experiment I: bedding content.

When keeping mice in cages, bedding is important for the animal's well-being. It keeps them warm, and they could use it for nesting material. In addition, it provides a comfortable cage bottom. CR mice tend to eat more bedding than ad-lib mice do (107). They eat about 1g of bedding per day if kept CR.

Mice were divided into three main groups: Ad-lib, CR, and overnight fasting (ON) (Figure 3). Within these groups three bedding types, wood, cellulose and corncob were used. ON contains two further groups: ON Grids (ON-G) and ON Empty (ON-E) (Figure 3). Mice tend to eat their own faeces which is known as coprophagy. In group ON-G, a grid prevented mice from access to cage bedding and eat their own faeces. Whereas in the ON-E group cage bedding was absent but coprophagy was possible. In each group, there were 10 mice.

Concerning CR, mice were given 75% of the normal food intake for two weeks. On the 11th day of CR, the weight of cage bedding was measured daily for three consecutive days to observe

the amount the mice eat. Regarding ON, mice had to fast for 16h since food was taken away in the evening with free access to water. Concerning ad-lib groups, the standard diet they had, was taken away two hours before the dissection (107).

2.3.2 Experiment II: Antibiotics and Faecal Transplant

For this group, 8-10 mice were randomly separated into the experimental groups:

- Ad-lib: standard chow, *ad libitum*
- CR: standard chow, 75% reduction of daily food intake
- AT ad-lib: standard *ad ad libitum* chow, antibiotics treated mice
- AT-CR: standard CR chow, antibiotics treated
- FT ad-lib: standard *ad libitum* chow fed mice treated with the microbiota transplant of ad-lib mice
- FT-CR: standard *ad libitum* chow fed mice treated with the microbiota transplant of CR-mice

To obtain gut flora depletion, the experimental groups antibiotic-treated (AT) and faecal transplant (FT) were gavaged with a 200µl antibiotics cocktail. This mixture included: vancomycin 0.5 g/l, neomycin 1 g/l, ampicillin 1 g/l and metronidazole 1 g/l. Within the AT group mice were gavaged with this mixture three times within the 14 days of treatment whereas the FT groups were gavaged antibiotics twice. For faecal transplant fresh faeces were mixed with PBS, vortexed and centrifuged for 3 min at 1000 G. Afterwards, the mice were gavaged with the supernatant. 14 days after the first faecal transplant, mice were killed.

2.3.3 Experiment III: IF, FMD and KD

For the IF, mice had to follow an alternate day fasting cycle, 24h ad-lib consuming standard chow and 24h of fasting. They had access to water *ad-libitum* the whole time.

For the FMD treatment, the mice had a 4-day intervention, followed by 7-days of refeeding. The fasting-refeeding cycle was repeated three times. On the first day of the fasting intervention, mice got a mix of broth powder, extra virgin olive oil, essential fatty acids, a green vegetable mix and hydrogel. The animals got 50% of the regular caloric intake which was 5,8 kcal per day. On the following days (2-4) a mixture of broth powder, glycerol and hydrogel was part of their diet. They received 10% of normal caloric intake, 1,2 kcal per day.

Regarding the KD, three different groups, two ketogenic groups and one control group were formed (Table 1). The control consisted mainly of starch and protein whereas the ketogenic diets had halved the amount of protein and no starch. The first ketogenic diet LCT consists of 100% LCT which is comparable with CKD. The other KD, LCT/MC, composition goes in the direction of an MCT-ketogenic diet. The MCT content, which is 34%, follows a typical 60-40 % distribution of caprylic and capric acid (Table 2).

Table 2: Composition of the ketogenic diet group; C8 = caprylic acid, C10 = capric acid, LCT = long chain triglyceride, MCT = medium chain triglyceride.

	KD Control	**KD LCT**	**KD LCT/MCT**
Description	16% protein	100% LCT (8.2:1)	66% LCT & 34%MCT (MCT =60%C8, 40% C10)
Crude Protein %	16,1	8,1	8,1
LCT %	0,8	74,6	49,6
C8 %	0	0	15,0
C10 %	0	0	10,0
Sugar %	6	1,0	1,0
Starch %	51,2	0	0
Crude fiber%	10,0	9,9	9,9
Crude ash%	4,4	4,4	4,4

2.3.4 Experiment IV: Taurine supplements

Besides the groups ad-lib and CR, taurine supplemented diets and fibre influence was part of this experimental group. Three different taurine diets were implemented and carried out with either ad-lib or CR. Taurine (Tau), low taurine diet (LTD) and normal taurine diet (NTD).

For the experiment "Tau", 5% taurine was added to the drinking water of mice, which were consuming standard chow. The LTD did not contain taurine, whereas in the NTD 0,5% taurine was added (Table 3). Both groups, LTD and NTD, did not contain cysteine or methionine. Standard chow includes a small amount of methionine and cysteine.

Table 3: Composition of LTD and NTD.

	Low taurine diet (LTD)	**Normal taurine diet (NTD)**
Casein	10	10
Cornstarch	52,3	51,8
Sucrose	10	10
Maltodextrin	12,5	12,5
Soybean oil	5	5
Fibre mix	3	3
Vitamin mix w/o choline	1	1
Mineral mix	6	6
Choline	0,2	0,2
Taurine	**0**	**0,5**

2.4 Measurement of the samples

2.4.1 Standard preparation

A standard curve is needed for calculating the concentration of an analyte in samples. Hence, at the beginning of our measurements mix-standard of all BAs was prepared and measured.

Standards are prepared by a dilution series. In order to prepare standard A (STD A), stock solutions of every BA were prepared. This means 1mg per each BA (TCA, LCA, CDCA, UDCA, TLCA, TDCA, DHCA, TUDCA and bile salts (CA/DCA)) was weighted in and mixed with 1 mL methanol (MeOH). Afterwards, STD 1 was prepared by mixing the BAs: 100µl of TCA, bile salts, LCA, CDCA, DHCA, TUDCA and UDCA, 20µl of TLCA and TDCA and

filled up with MeOH. Since 1000µl are required, 260µl MeOH were added to the BAs in STD 1. Then it follows a typical dilution series, taking 500µl of STD 1 and pipetting it into STD 2. After vortexing again, 500µl of STD 2 was pipetted in STD 3 (Figure 6). And so forth, except STD 6 which is also prepared of STD 4 (Figure 4).

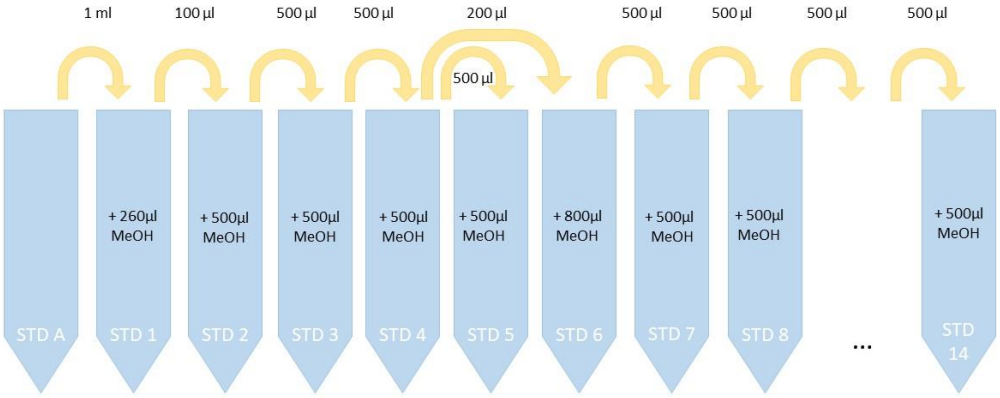

Figure 4: Dilution series.

After preparing the standards, they were transferred into an HPLC vial and STD 4-14 was measured with the LCMS.

Using the measured area under the curve (AUC) and the known concentrations of the standards, standard curves for every BA were created using Excel Microsoft®. The measured AUC of each STD 4-14 of our measurements, respectively, was given on the x-axis, whereas the y-axis contains the concentrations of every BA. The linear equation was calculated and could be used for the calculation of the samples' concentration.

2.4.2 Sample preparation

In the case of plasma, 50µl were transferred into a new Eppendorf. Except for faeces, more than 7mg were weighed in for the solid tissues (108). 7mg were too little since the extracted amount

was not measurable with LCMS anymore. Probably due to too much sample loss during the preparation steps since faeces samples are impure. more than 10mg of tissue was needed for faeces extraction.

Sample preparation differs between different body segments of mice. Regarding plasma samples, 50µl of plasma was mixed with 150µl of MeOH after defrosting them at room temperature. Afterwards, the plasma was shaken continuously for ten minutes with the laboratory rocker at 4°C. After centrifugation, for ten minutes at 4°C 12000 G, 100µl of the supernatant was transferred into a new Eppendorf and evaporated with the concentrator. The concentrator was set at 60°C and the samples were spun for 15 minutes. When evaporated completely, the samples were re-dissolved in 50µl MeOH (108).

Concerning intestinal samples, a water bath with 37°C and dry ice mixed with 70% ethanol had to be prepared. The sample should be at the tip of the Eppendorf so that thawing and freezing reach the sample. The frozen samples were held in the water bath for a few seconds and immediately put on dry ice for further few seconds afterwards. These thawing and freezing processes had to be carried out five times. Thereafter the samples got solved in MeOH with an amount being nine times higher than the sample's weight. For example, 10µl of the intestinal sample was mixed with 90µl MeOH. After vortexing, the samples got centrifuged at 12 000 G, at 4°C for 10 minutes and the supernatant was transferred again to a new Eppendorf. To be sure, that there are no impurities left, the intestinal samples were centrifuged another time (same settings) (108).

All other samples such as faeces, liver, muscle, kidney, heart, spleen, and brain were dissolved in a nine times higher amount of MeOH. The homogenisation of the samples was conducted with the Precellys®24 Tissue Homogenizer twice for 15 seconds at the lowest setting which is 4000 rpm. After centrifugation at 5000 G for 5 min, the supernatant was centrifuged again for 10 min at 12 000 G at 4°C. In case of impure tissues like faeces and cecum, another centrifugation step was needed (with the same setting as before). As far as the liver is concerned, another step of centrifugation was not necessary (108).

2.5 LCMS

For measuring BAs, the LCMS 8040 of Shimadzu® was used. The Liquid Chromatography Mass Spectrometry is a machine which combines HPLC and Mass Spectrometry. Mixing chromatography with mass spectrometry was urgently needed for the analysis of environmental samples, biological tissues, and fluids since the characterisation of such complex samples with a stand-alone analytical technique are practically impossible. The difficulty here is to identify the separated components with a compound specific detector which is ensured with mass spectrometry (109).

The LC-MS consists of three major components: a separation device (LC), an interface/ ion source and mass spectrometry (MS) (Figure 5) (109).

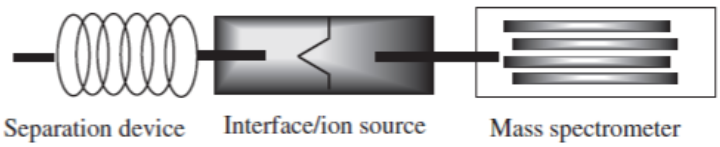

Figure 5: Three major components of the LCMS (109)

HPLC coupled with the MS is the mainstream method for detecting a mixture of BAs. The advantage of this technique is, that it is possible to distinguish between isomers with the same fragmentation pattern. separate isomers with the same ion fragments (110).

2.5.1 Liquid chromatography

For the measurements, the column Kinetex® 5 µm EVO C18 100 Å LC Column 150 x 3.0 mm was used. The column oven temperature was set to 30°C.

In the case of this thesis, the mobile phase consists of two different running media. Running medium A contains HPLC water, 0,1% formic acid, and 20mM ammonium acetate. The medium was filled in a bottle and shaken till the ammonium acetate was solved. Medium B was mixed from a 3:1 ratio of ACN to MeOH, 0,1% formic acid as well as 20mM ammonium acetate. In a 1L bottle, this would result in 750mL ACN, 250mL MeOH, 1000µL formic acid and 1,54g ammonium acetate.

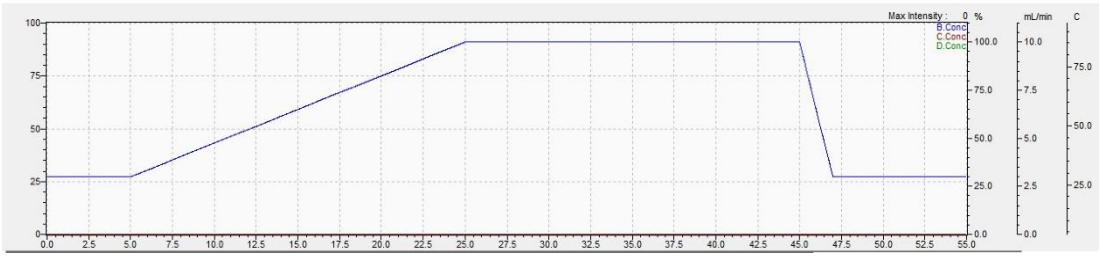

Figure 6: Running medium B.

Initially, 30% B was achieved (and 70% A) which was held for 5 minutes. Then B rose to 100%. At minute 25, 100% of the medium B was reached which stayed for 20 minutes. Afterwards, it was set back to the initial 30% B within 2min, followed by 10 minutes of re-equilibration (Figure 6).

The autosampler, for injecting the samples, was set to 4°C. Due to the reverse phase coating of the column, non-polar BAs will prefer to remain longer in the column and therefore have higher retention times compared to more polar BAs.

2.5.2 Mass spectrometry

In contrast to liquid chromatography, MS provides a highly sensitive detection technique. The MS consists of an ion source for ionizing the samples, an ion guide which is an electrostatic lens, a mass analyser for separating ions based on the mass to charge ratio (m/z) and additionally a detector for the detection of the separated ions (111).

In the case of the Shimadzu 8040 LCMS, an MS/MS is used which is called a hybrid or tandem MS. The ions are separated in the first analyser, then enter the collision cell and undergo fragmentation. The results are so-called product ions which are then separated in the second mass analyser and afterwards detected. Hybrid mass spectrometry is specifically used for drug discovery, polysaccharides, protein or metabolomics identification (111).

2.5.3 Analysis of the LCMS data

For each BA arises a peak at the computer program with the calculated AUC. For analysis of the data, the expected retention times for each BA are used, to define the right peaks. When all

the AUCs for the BAs are collected, Excel Microsoft® was used for further calculations. To get the exact concentration of each BA within the samples, the linear equitation y=ax+b was used. Together with the measured standards, the concentration for the BA was calculated. For statistical tests, a paired sample t-test in Excel Microsoft® was carried out. p-value was <0,05 and additionally, Bonferroni correction was used.

2.6 Polymerase chain reaction (PCR)

For qPCR measurements, the Quant studio™ 6 Flex Real-time PCR System from Life Technologies Holding was used.

The real-time qPCR is a combination of the qPCR (quantitative PCR in real-time) and the RT-PCR (reverse transcription PCR). Regarding reverse transcription, RNA is used as a template to receive cDNA (complementary DNA) which gets further converted into double-stranded cDNA and put into a standard PCR amplification. qPCR is described as an amplification of DNA in real-time measured by a fluorescent probe (112).

2.6.1 RNA Extraction

For RNA extraction the RNA Kit "peqGOLD HP RNA Kit" from VWR International GmbH was used.

For the RNA Extraction around 10mg of tissue was weighed in and put into an Eppendorf. Additionally, the DNA removing tubes (green tubes), the RNA binding tubes (red tubes), the collection tubes and normal Eppendorfs were prepared for the extraction (Figure 7).

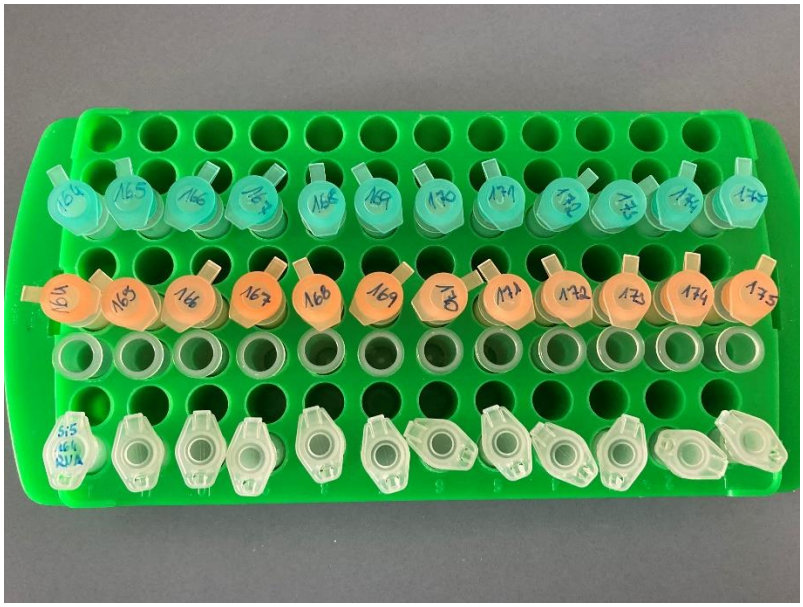

Figure 7: Preparation step for the RNA Extraction. green tubes = RNA removing tubes, red tubes = RNA binding tubes, collection tubes and Eppendorfes.

The first step was to dissolve the tissue in 200µl lysis buffer. Therefore, the buffer was added to the Eppendorf and a syringe was used for better dissolution. By pulling the sample up and down in the syringe tip, the sample could be dissolved well. Afterwards, the dissolved sample was transferred into the DNA removing tube and put on normal ice. The samples were centrifuged at 12 000 G at 20°C for 1 minute. For the next step the centrifuged liquid was mixed with 200µl (same amount as lysis buffer) 70% EtOH and slowly mixed within the tip. Subsequently, the mix was immediately transferred to the RNA binding tube and centrifuged again at 10 000 G, 20°C for 1 minute. After centrifugation, the collection tube was renewed. For the washing step 500µl of washing buffer I was added, and the sample was spun down at 10 000 G, 20°C for 1 minute. The centrifuged liquid is then emptied and tapped out. When tapped out, the sample is ready for washing buffer II, which contains 100% EtOH. After adding 500µl of buffer II the washing step is repeated the same way as before. After centrifugation, the washing step with buffer II is repeated. After 3 washing steps, the samples were dried within the centrifuge for 2 minutes.

For the elution, 50µl RNAse free water was added directly on the membrane of the RNA binding tubes. Therefore, the previous collection tubes were replaced by Eppendorfes. The samples are centrifuged at 6000 G and then the RNA is completed. Before the samples are

applied to the NanoDrop™ One, the NanoDrop has to be calibrated with RNAse free water. After blanking the samples, they could be measured with the NanoDrop™ for measuring RNA wavelength.

2.6.2 Reverse transcription

To extract cDNA, the first step was to pipette a specific µl amount of RNAse free water into a new Eppendorf. During this step, the RNA samples had time to taw, and the heating blocks could reach their needed temperature. Then a specific µl amount of the RNA samples were pipetted into the Eppendorf with RNAse free water. To each sample 5µl of master mix (MM) were added before spinning down with the microcentrifuge. The MM consisted of 4µl buffer and 1µl reverse transcriptase per sample. To ensure that there was enough MM, 5% more was produced. After adding MM to the samples, three different heating blocks with different temperatures were used. First, the samples were heated for 5 minutes at 22°C, then for 30 minutes at 42°C and the last step was again 5 minutes at 85°C. For cooling down, the samples had to rest for 5 minutes at room temperature. Afterwards, 380µl RNAse free water was added, and the Eppendorf was tipped and put on ice. After vortexing the samples, they were frozen at -20°C and ready for pipetting on a qPCR plate.

2.6.3 Preparation of the qPCR measurement

When pipetting the cDNA onto the qPCR plate, the plate was put on ice. Samples were pipetted following a pipetting scheme. The wells were filled with 2µl of cDNA and 8µl MM. For the MM SYBR Green, the forward and the reverse primer and RNAse free water were mixed (Table 4).

Table 4: MM preparation. For each well 8µl MM is required. By multiplying the amount by the number of wells the total MM could be calculated.

in µl	multiply with number of wells
SYBR Green	5
primer forward	0,3
primer reverse	0,3
RNAse free water	2,4

After preparing the MM and pipetting it onto the plate, the plate got centrifuged at 1000 G for 1 minute and sealed. The plate was then ready for the qPCR measurement.

2.6.4 qPCR measurement

The structure of the machine consists essentially of 3 parts: a thermal cycler with an integrated excitation light source, a fluorescence detection system and a software, that shows the recorded fluorescence data as a DNA amplification curve (113). Concerning fluorescent DNA dye, SYBR® Green is the commonly used dye (113) and is also part of the measurements of this master thesis.

In the beginning, when amplification starts, the fluorescence level is low. If the reaction progresses into the exponential phase, the fluorescence level rises and increases above the baseline. This level is called the threshold level (112). The threshold cycle (Ct) represents the number of cycles that are needed that the fluorescence crosses the baseline level (114). In terms of relative quantitation, the ratio between a reference gene (RG) and the gene of interest is calculated. The so-called RG is a control present in all samples with a stable concentration (112).

For analysis, the delta-delta Ct method (ΔΔCt) is applied which describes the ratio between RG and the gene of interest. Therefore, first, a ΔCt value is created, which is the Ct of the reference gene subtracted from the Ct of the gene of interest. The ΔCt value is then compared to a control sample to receive the ΔΔCt. (112).

In the case of this thesis the reference gene Eef1a1, Eukaryotic translation elongation factor 1 alpha 1 was used.

3 Results

3.1 Experiment I – Impact of bedding, ON fasting and coprophagy

3.1.1 Impact of bedding type on BAs

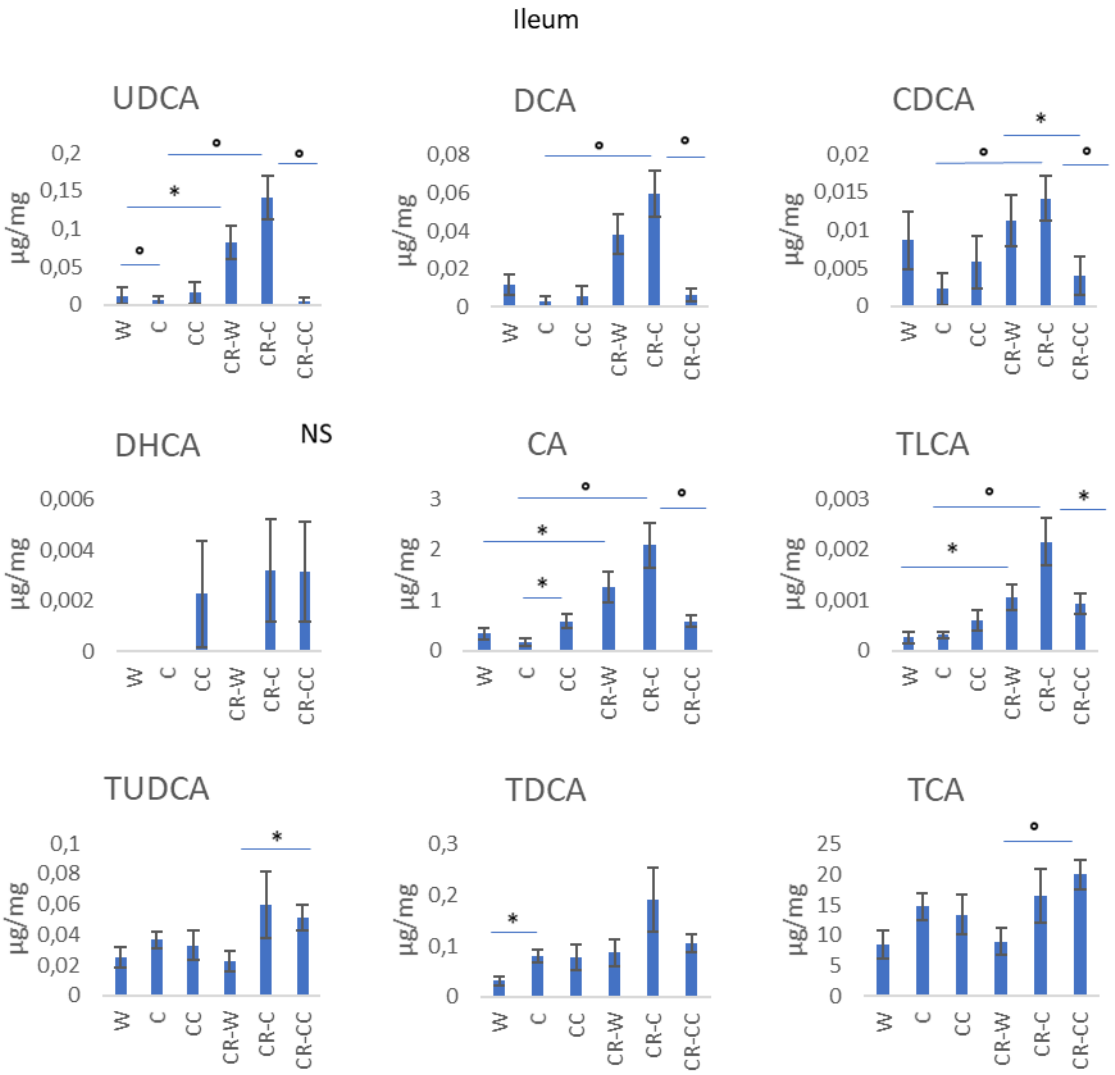

*Figure 8: BAs were measured in ileum. Statistical significances are marked with *p<0,05 and °p<0,0084 (after Bonferroni correction). W= wooden; C = cellulose; CC = corncob; CR = caloric restriction.*

All nine measured BAs in the ileum show, that CR mice housed on cellulose bedding have the highest BA concentration. Furthermore, it can be observed that CR in general leads to higher BA concentrations than ad-lib does (Figure 8).

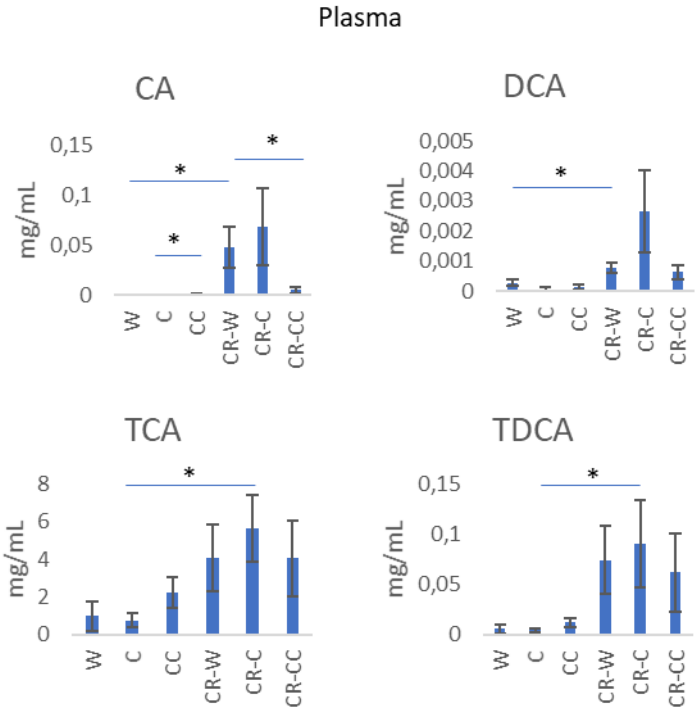

Figure 9: CA, DCA, TCA and TDCA were measured in plasma. Statistical significances are marked with *p<0,05 and °p<0,0084 (after Bonferroni correction).

This observation is also true for plasma, highest BA concentration was measured within the CR-C group (Figure 9). In faeces, however, cellulose (ad-lib fed mice) led to the highest BA excretion (Figure 10).

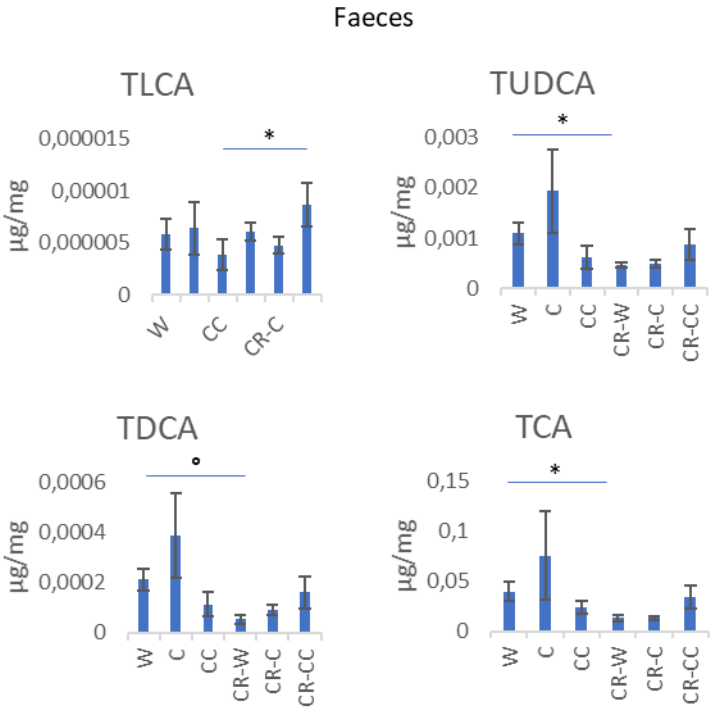

*Figure 10: Taurine conjugated BAs were measured in faeces. Statistical significances are marked with *p<0,05 and °p<0,0084 (after Bonferroni correction).*

3.1.2 Influence of coprophagy on BAs

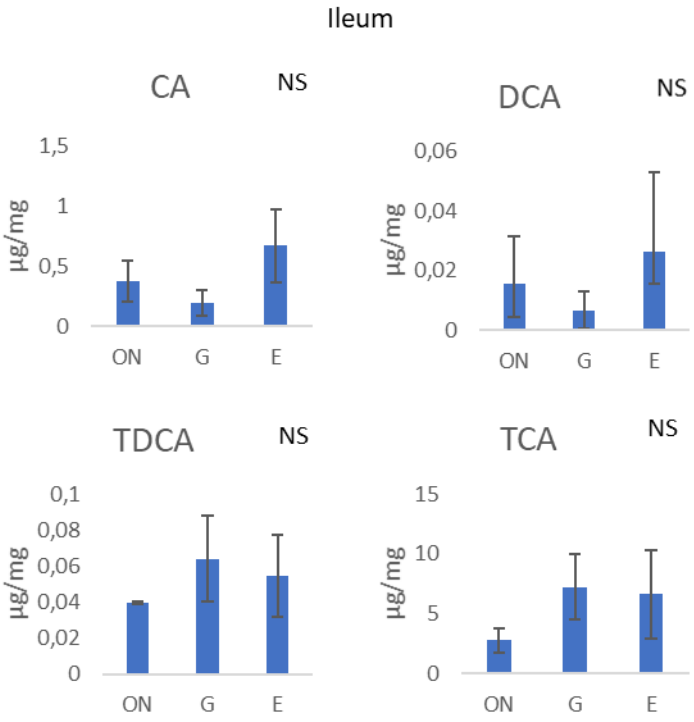

Figure 11: CA, DCA, TDCA, TCA concentrations were measured in ileum. Comparison between Empty, Grid and Overnight-Wood. No significances were found (=NS).

Regarding the intervention groups E, G and ON, no significance was found. However, there was a tendency for unconjugated BAs CA and DCA in the group E. Mice without bedding, but with coprophagy had higher levels of the unconjugated BAs. On the contrary, taurine-conjugated BAs did not show this effect (Figure 11).

3.1.3 FGF15 and IBAT results

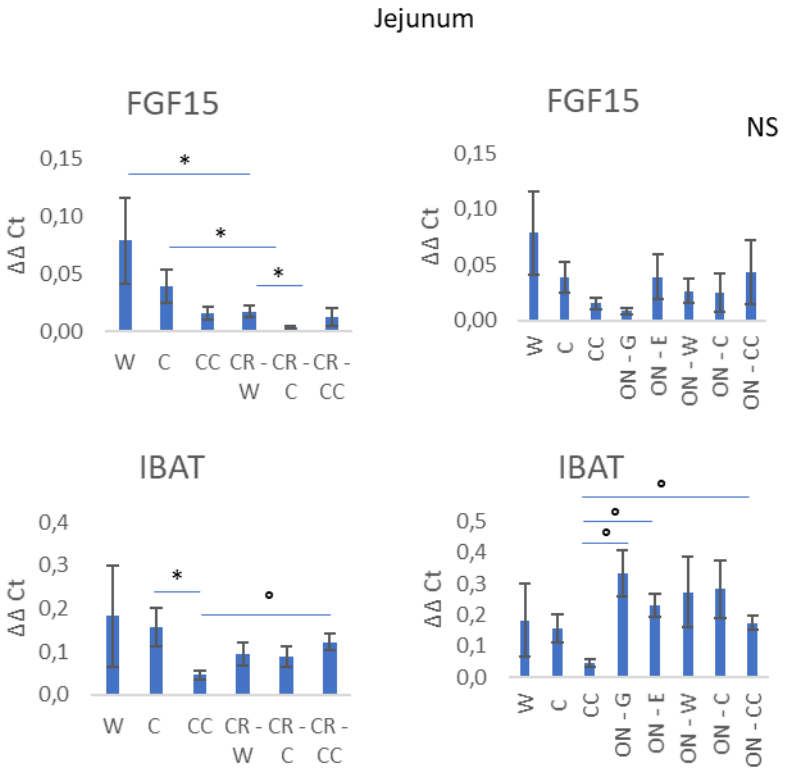

*Figure 12: FGF15 and IBAT expressions in jejunum compared between ad-lib and CR as well as ad-lib and ON. Statistical significances are marked with *p<0,05 and °p<0,0084 (after Bonferroni correction) for ad-lib vs. CR and °p<0,0063 for ad-lib vs. ON. ΔΔCt = delta delta Cycle threshold.*

FGF15 was higher expressed during ad-lib feeding, in comparison with the CR group. This relation is significant. Concerning IBAT expression, controversial ΔΔ- Ct values were measured. It seems as if ON fasting without coprophagy led to the highest expression in IBAT. Moreover, ON results in higher IBAT expression, whereas, when comparing ad-lib bedding with CR bedding, the ad-lib group results in higher levels. FGF15 expression is most influenced by the bedding wood, also IBAT seems to be most affected by the wooden bedding (Figure 12).

3.2 Experiment II – Impact of AT and FT

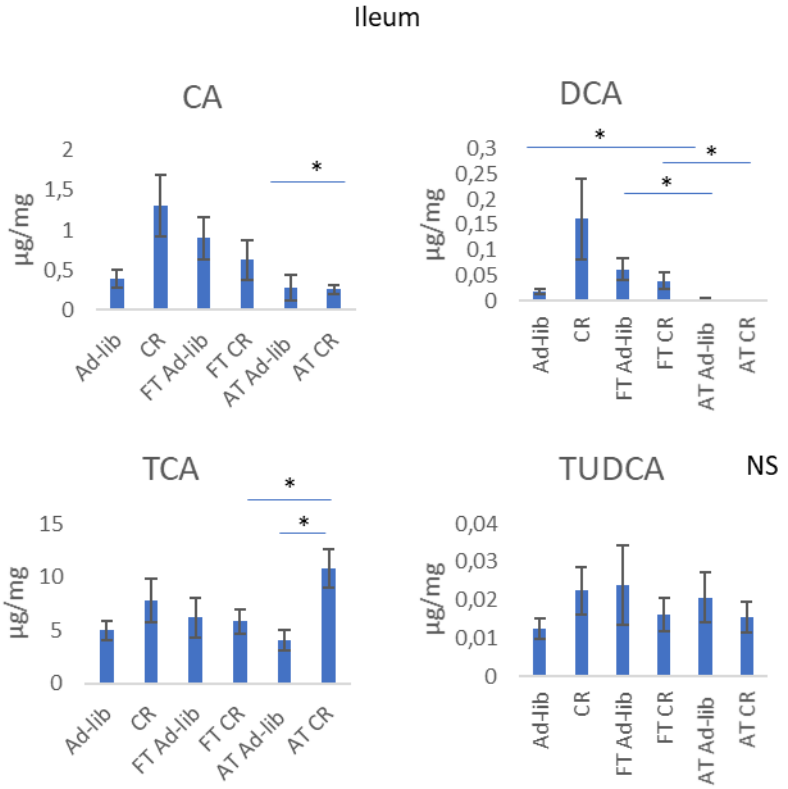

*Figure 13: BAs CA, DCA, TCA and TUDCA measured in ileum. Statistical significances are marked with *p<0,05 and p°<0,0084 (after Bonferroni correction). Ad- lib = ad libitum; CR= caloric restriction; FT = faecal transplant; AT= antibiotics*

The assumption that more BAs are produced during CR, could be shown again in higher concentrations in ileum, plasma and liver (Figures 13, 14, 15). In faeces, however, the ad-lib group shows higher BA levels (Figure 16).

Mice with AT show no or hardly any secondary BAs as seen here in the BA DCA. This can also be proved in the liver and plasma (Figures 13,14,15).

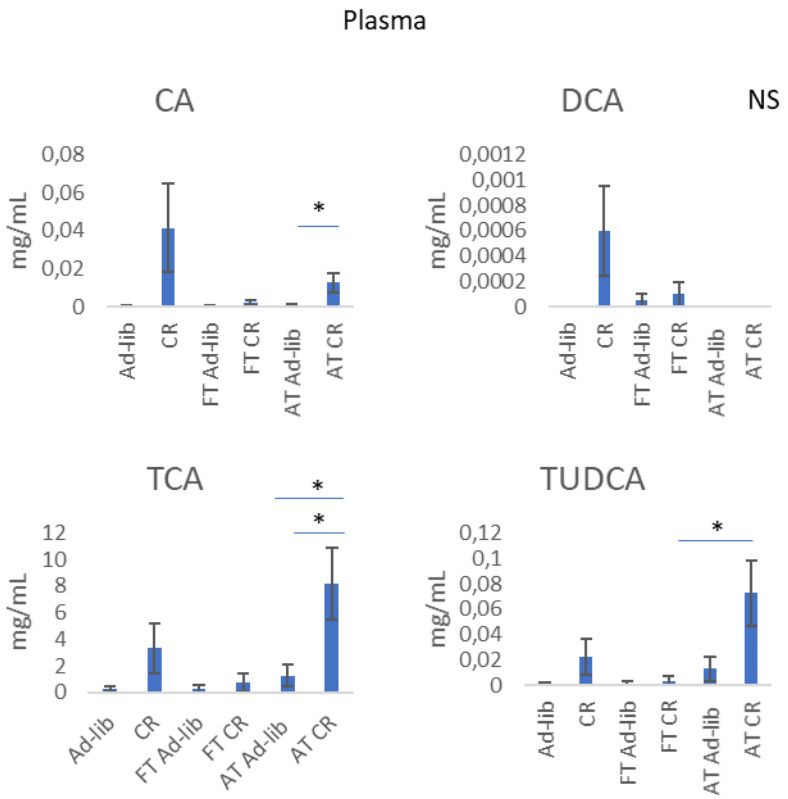

*Figure 14: CA, DCA, TCA and TUDCA measured in plasma. Statistical significances are marked with *p<0,05 and °p<0,0084 (after Bonferroni correction).*

In plasma and liver, it could be observed, that CR mice treated with antibiotics show higher levels of CA (Figures 14, 15), whereas in faeces the AT ad-lib group shows higher concentrations of CA (Figure 16).

Mice that received FT of CR mice (FT-CR), do not show the same rise of BAs as the CR group in the ileum, liver and plasma (Figures 13,14,15). Hardly any differences can be detected.

Additionally, in plasma, DCA was not measured if AT was applied (Figure 14).

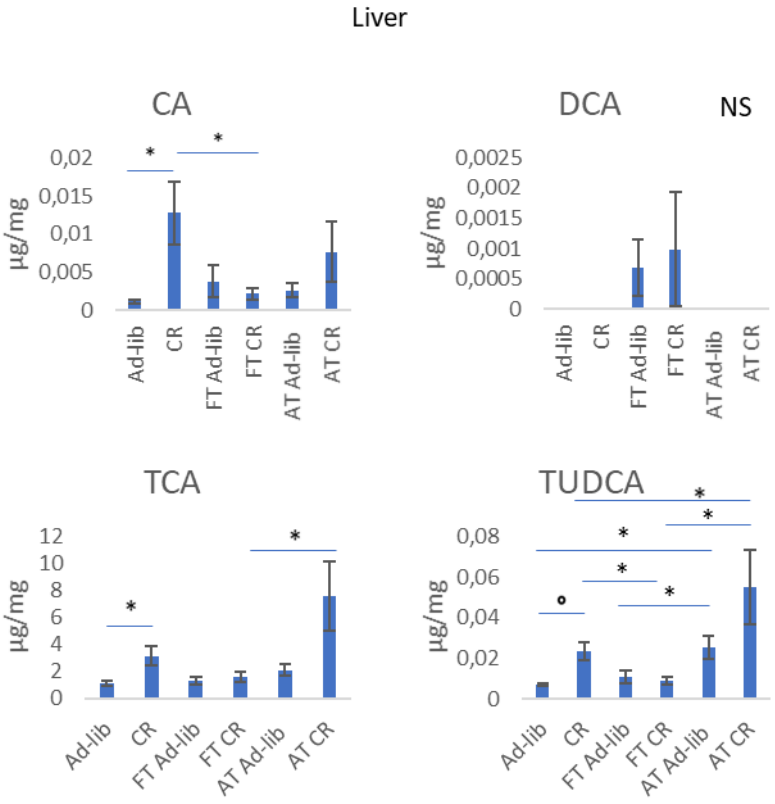

*Figure 15: CA; DCA, TCA and TUDCA measured in liver. Statistical significances are marked with *p<0,05 and °p<0,0084 (after Bonferroni correction).*

Another finding shows that the AT group in primary taurine-conjugated BAs (TCA, TUDCA) shows much higher levels compared to unconjugated BAs (DCA, CA) in liver, plasma and ileum (Figures 13,14,15). The concentration of TCA in the ileum under CR is even higher in AT mice (Figure 13).

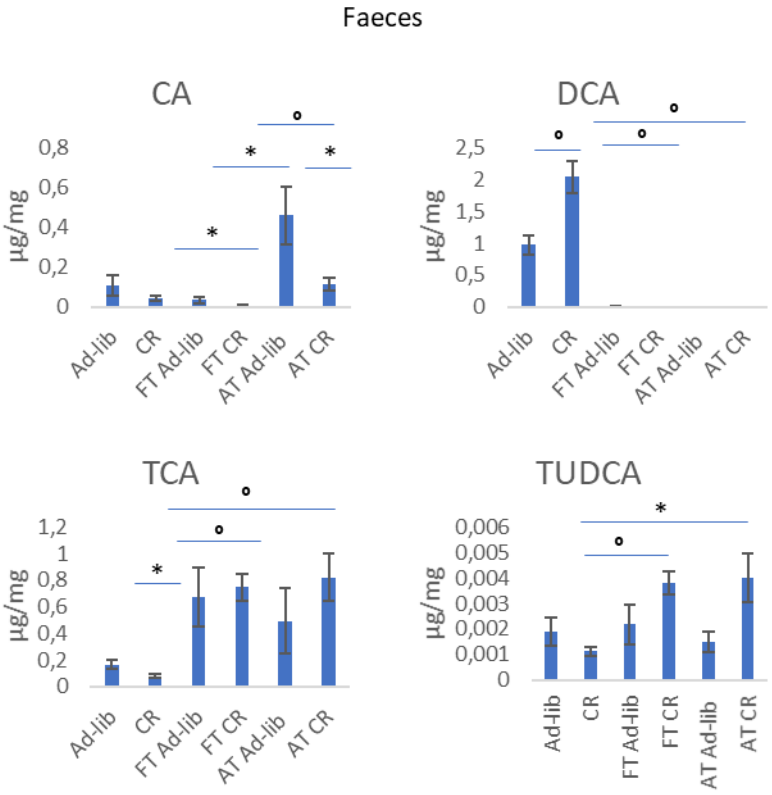

*Figure 16: CA, DCA, TCA and TUDCA measured in faeces. Statistical significances are marked with *p<0,05 and °p<0,0084 (after Bonferroni correction).*

In faeces, ad-lib rises higher BA concentrations than CR does. However interestingly, not for DCA. This BA shows a higher concentration during CR. For AT and FT results are controversial. AT ad-lib leads to the biggest increase of CA, whereas the AT CR group impacts TCA and TUDCA even more (Figure 16).

3.2.1 qPCR results

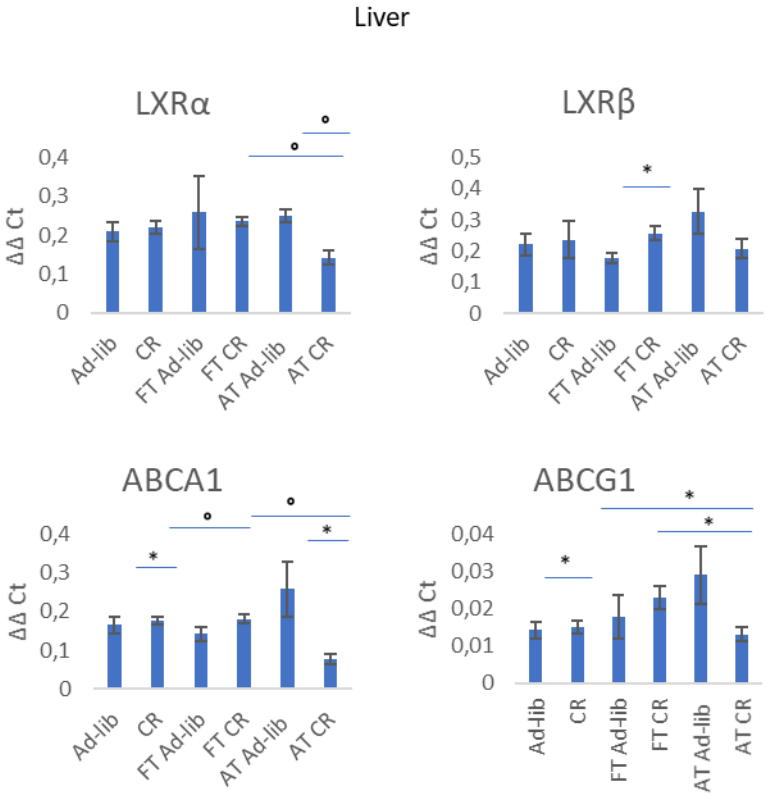

*Figure 17: qPCR results for LXRα, LXRβ, ABCA1 and ABCG1 Statistical significances are marked with *p<0,05 and °p<0,0084 (after Bonferroni correction).*

LXRs as well as the ATP binding cassette transporters do not show any clear differences between ad-lib and CR. LXRβ shows slightly higher expression levels in the CR group. Both cassette transporters are slightly higher expressed during CR, which is also statistically significant (Figure 17).

All four, the liver x receptors and the cassette transporters seem to get especially activated via the AT ad-lib group. Since LXR α/β affects ABCG1 and ABCA1 (79), it could be expected that the expressions of both should run the same way (Figure 17).

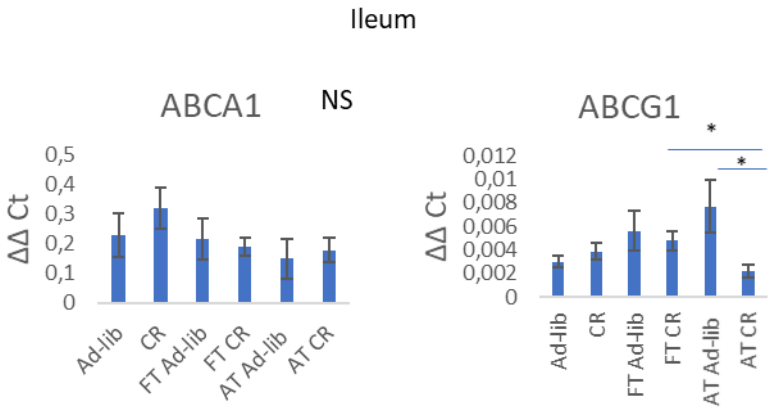

*Figure 18: ABCA1 and ABCG1 expressions in the ileum. Statistical significances are marked with *p<0,05.*

In the ileum, CR leads to higher expressions of ACBA1 and ABCG1 than ad-lib. AT ad-lib still leads to higher expressions in the ABCG1 but not in the ABCA1 transporter (Figure 18).

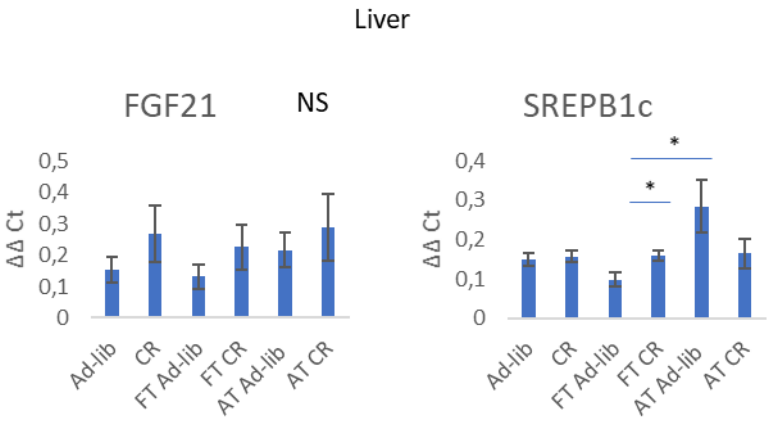

*Figure 19: FGF21 and SREBP1c expressions in liver. Statistical significances are marked with *p<0,05.*

The fasting effect of FGF21 can be supported. CR, as well as FT-CR and AT-CR, lead to higher FGF21 levels in the liver. SREBP1c is mostly impacted by the AT ad-lib group, which is comparable to the receptors LXRα/β and the transporters ABCA1 and ABCG1. Comparing ad-lib with CR, CR leads to a higher expression (Figure 19).

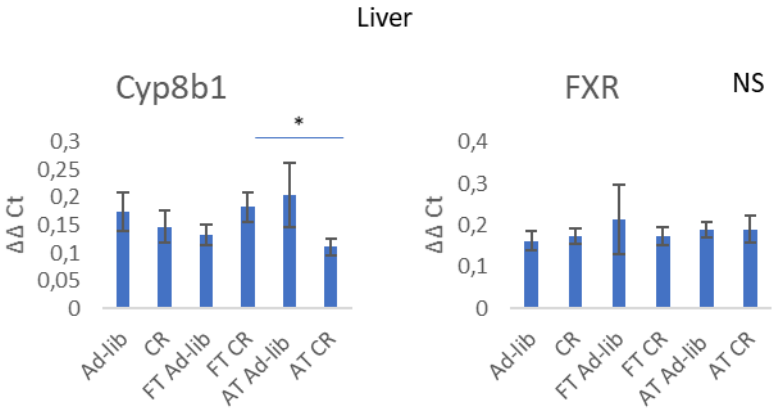

*Figure 20: CYP8B1 and FXR expressions in liver. Statistical significances are marked with *p<0,05.*

For FXR in the liver, neither significances nor differences are detectable. Concerning CYP8B1, a rise in the group AT ad-lib, as well as FT-CR and ad-lib was measured. It is mainly stronger expressed by ad-lib groups (Figure 20).

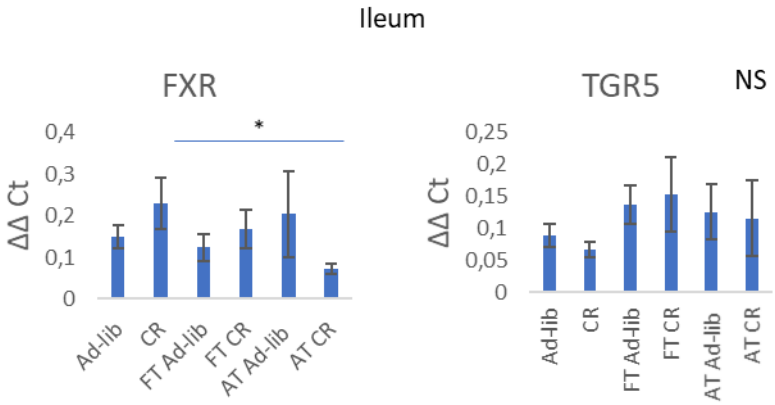

*Figure 21: FXR and TGR5 expression in ileum. Statistical significances are marked with *p<0,05.*

Whereas in the liver no FXR changes are identified, there are clear differences in the ileum. CR, FT-CR as well as AT ad-lib lead to higher expressions. However, there is no statistical significance between ad-lib and CR. For the TRG5 gene hardly any differences are visible, higher expressions are found in the FR-CR group, however, without significance (Figure 21).

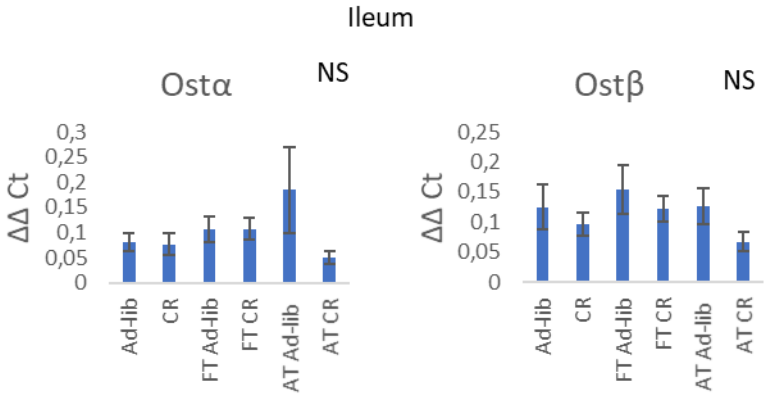

Figure 22: Ostα and Ostβ expressions in ileum. No significance was measured.

The efflux transporters Ostα/β do not show significant differences between CR and ad-lib. The α-transporter is mostly expressed during AT ad-lib, whereas the β-transporter is higher expressed during the FT Ad-lib treatment. Ad-lib applications had a bigger impact on the efflux transporter; however, the differences are negligible (Figure 22).

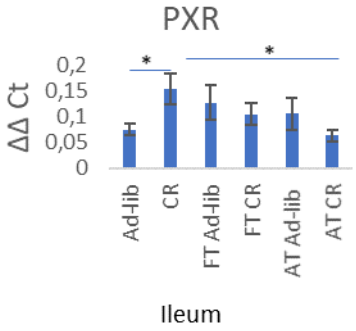

*Figure 23: PXR expression in ileum. Statistical significances are marked with *p<0,05.*

PXR is mainly expressed during CR and interestingly shows high levels in the groups AT ad-lib and FT ad-lib (Figure 23).

3.3 Experiment III: KD, IF and FMD

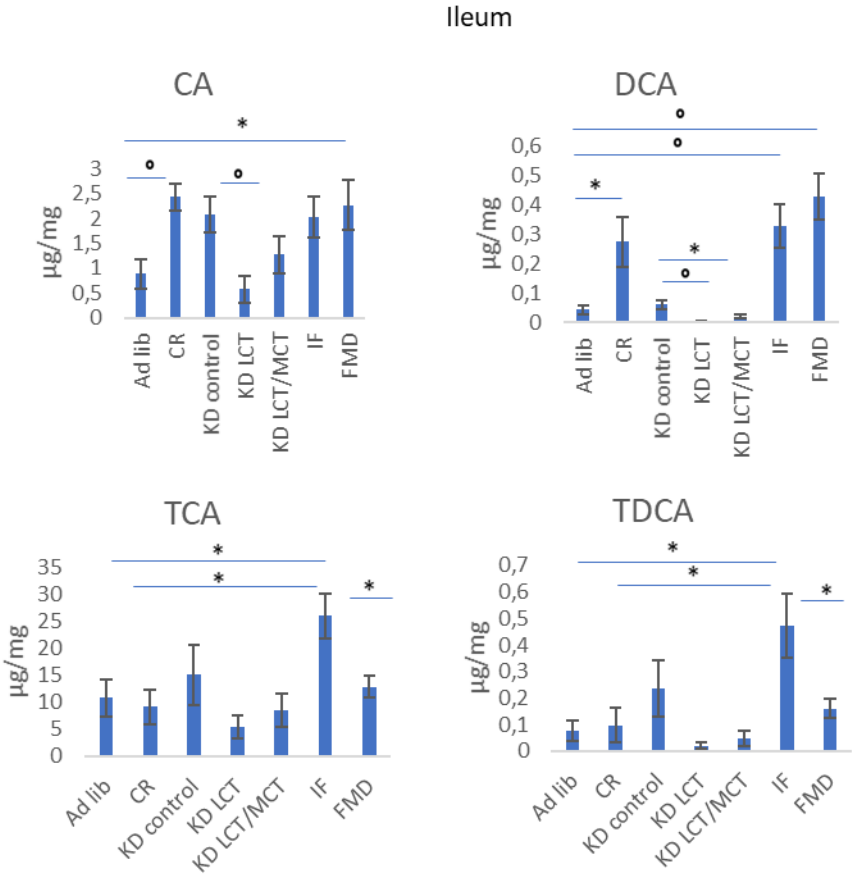

*Figure 24: CA, DCA, TCA and TDCA measured in ileum. Statistical significances are marked with *p<0,05 and °p<0,0084 (after Bonferroni correction). Ad-lib = ad libitum; CR = caloric restriction; KD control= Ketogenic control group; KD LCT= ketogenic diet with LCT; KD LCT/MCT = ketogenic diet with LCT/MCT; IF = intermittent fasting; FMD = fasting mimicking diet.*

Besides CR, FMD and IF also lead to higher BA concentrations in the ileum. IF especially affects taurine-conjugated BAs, whereas FMD has the biggest impact on unconjugated BAs (CA, DCA, CDCA, UDCA). Regarding the ketogenic diet, the control group (KD control) has shown the highest BA concentrations. The BAs CA and TCA are more influenced by the keto diets than all the other BAS. However, the control group still shows the highest levels. If comparing the two KDs with each other, KD LCT/MCT shows slightly higher BA concentrations (Figures 24, 25).

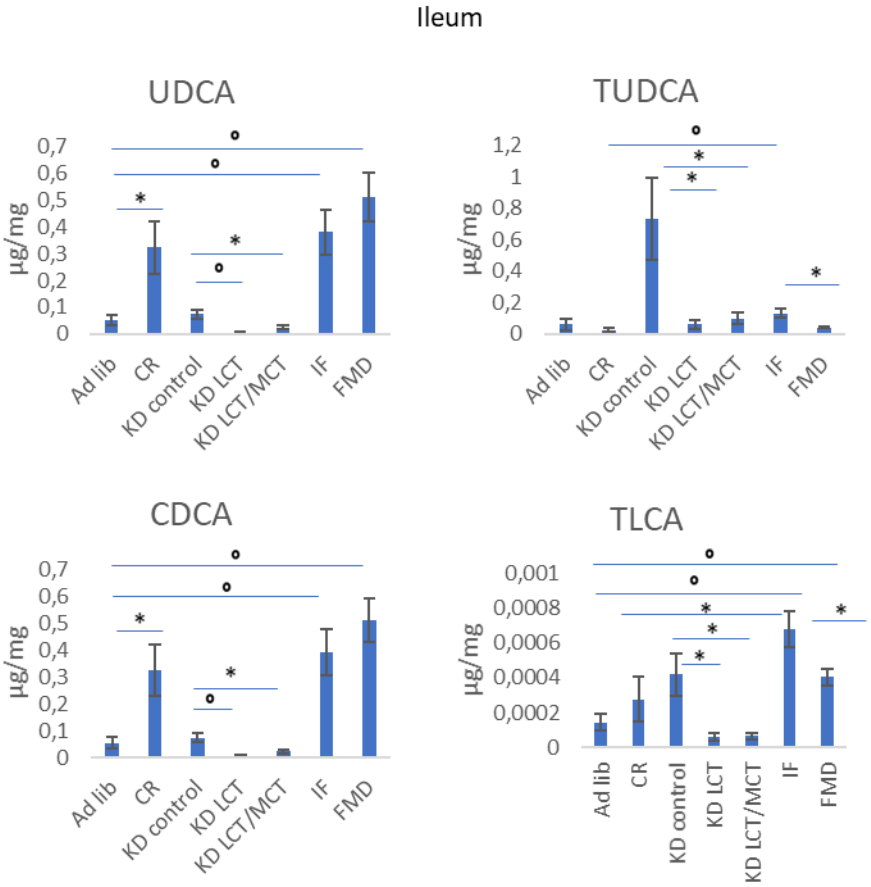

*Figure 25: UDCA, TUDCA, CDCA and TLCA measured in ileum. Statistical significances are marked with *p<0,05 and °p<0,0084 (after Bonferroni correction).*

3.4 Experiment IV

3.4.1 Taurine diets

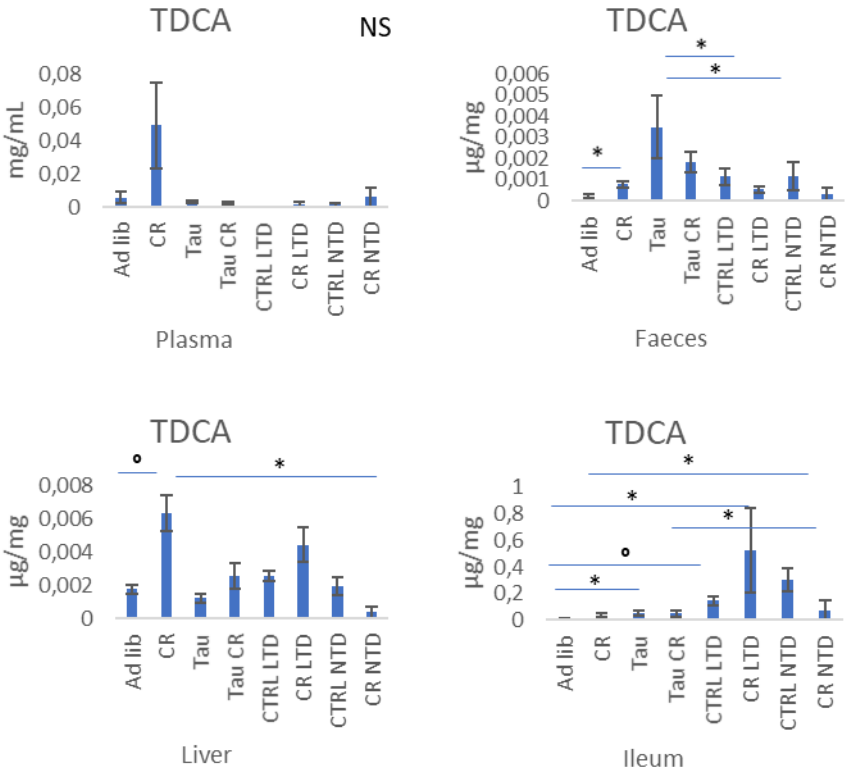

*Figure 26: Taurine diets in taurine-conjugated BA TDCA. Statistical significances are marked with *p<0,05 and °p<0,00625 (after Bonferroni correction). Ad-lib = ad libitum, CR = caloric restriction, Tau = taurine, Tau CR = taurine caloric restriction, CTRL LTD = control low taurine diet, CR LTD = caloric restricted low taurine diet, CTRL NTD = control normal taurine diet and CR NTD = caloric restriction normal taurine diet.*

In plasma, liver and ileum CR leads to an increase in BA levels. CR mice have higher levels of taurine-conjugated BAs as seen here in TDCA and TCA.

Interestingly, after the application of taurine diet forms (Tau, NTD, LTD) hardly any concentrations for BAs can be measured in plasma. Only the CR-NTD has slightly higher concentrations. If comparing liver and ileum, higher concentrations were measured in the ileum (Figures 26, 27).

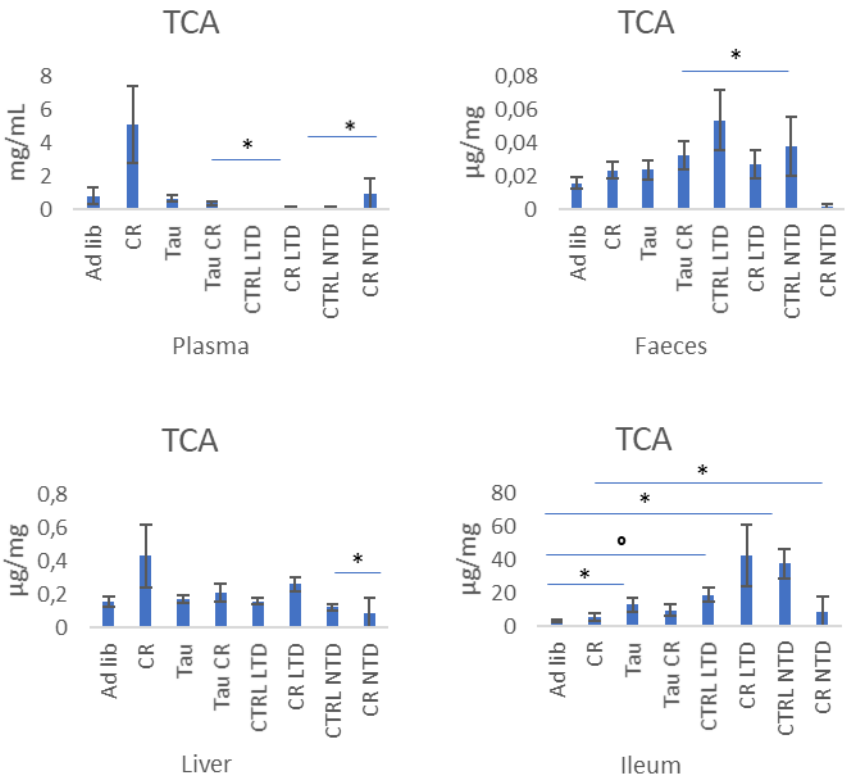

*Figure 27: Taurine diet content in taurine-conjugated BAs TCA. Statistical significances are marked with *p<0,05 and °p<0,00625 (after Bonferroni correction).*

In ileum, liver and plasma all CR forms of the taurine diet lead to higher BA concentrations, whereas in faeces the control group of this taurine diet resulted in higher BA levels. Moreover, CR LTD in the ileum and liver has the biggest impact on taurine-conjugated BAs among all the taurine diet forms. Regarding ileum, there is an exception in the taurine effect "pattern", the CTRL NTD leads to a bigger rise in taurine-conjugated BAs than CR-NTD (Figures 26, 27).

3.4.2 qPCR results within the taurine diet group

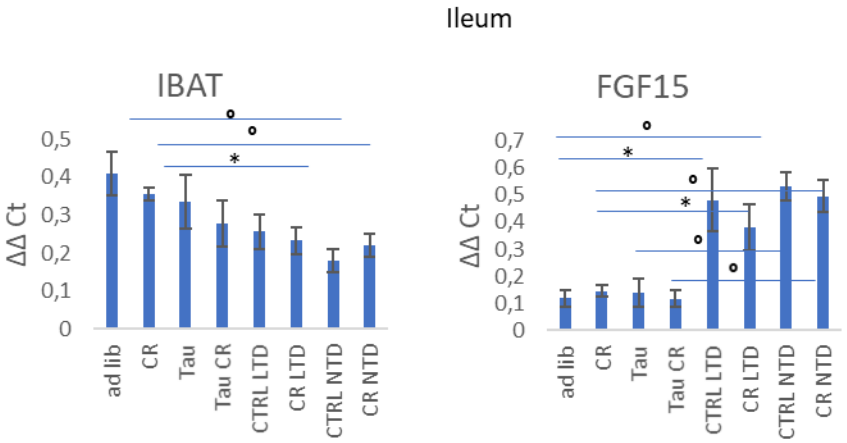

*Figure 28: IBAT and FGF15 measured in ileum. Statistical significances are marked with *p<0,05 and °p<0,00625 (after Bonferroni correction).*

As already seen in the former IBAT measurements, the results are controversial and not conclusive. Ad-lib leads to the biggest expression, and also Tau results in high ΔΔCt values. Concerning FGF15 ad-lib does not lead to higher expressions than CR but in the other taurine-ad-lib diets higher expressions of Tau, control LTD and control NTD can clearly be stated (Figure 28).

3.5 Organ comparison

To find out, whether BAs are also circulating in other tissues and affected by fasting, further tissues were measured. Spleen, kidney, muscle, heart, eWAT (epididymal white adipose tissue) and brain were collected and investigated for BAs.

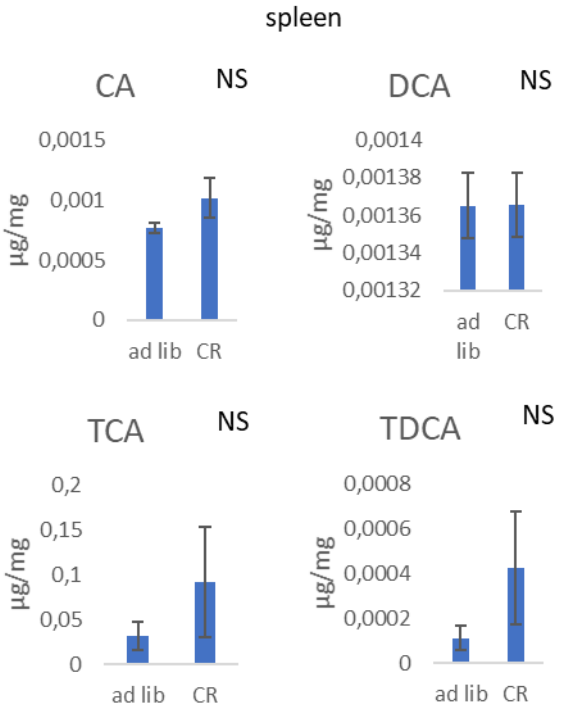

Figure 29: CA, DCA, TDCA and TCA concentrations measured in spleen. No significances were measured. Ad-lib = ad libitum, CR = caloric restriction.

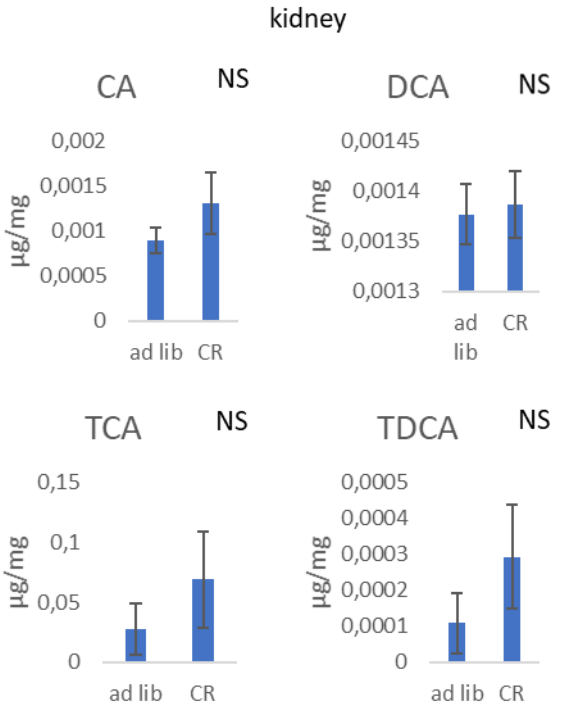

Figure 30: CA, DCA, TDCA and TCA concentrations measured in kidney. No significances were measured.

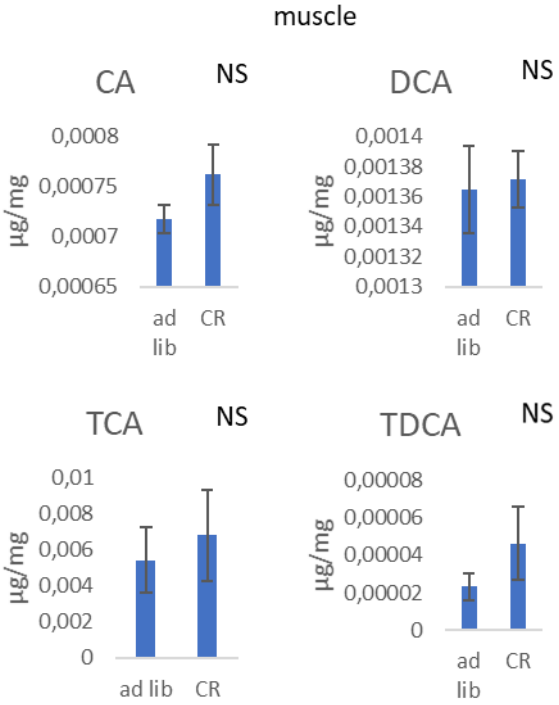

Figure 31: CA, DCA, TDCA and TCA concentrations measured in muscle. No significances were measured.

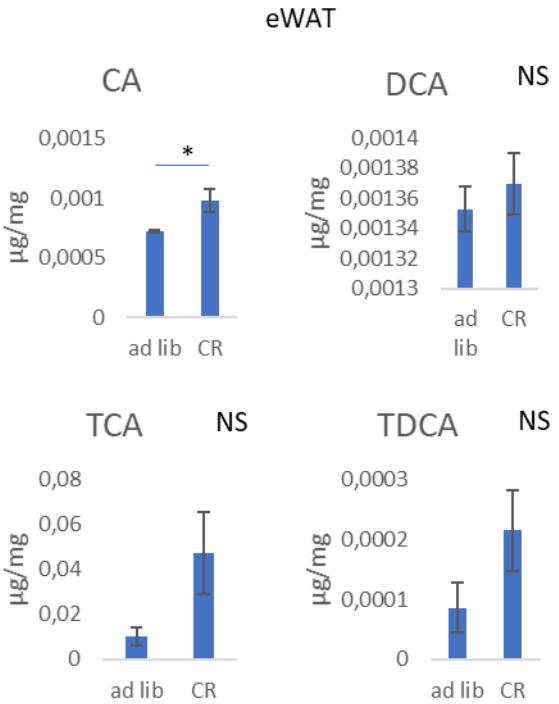

*Figure 32: CA, DCA, TDCA and TCA concentrations measured in eWAT. Statistical significances are marked with *p<0,05. eWAT = epididymal white adipose tissue.*

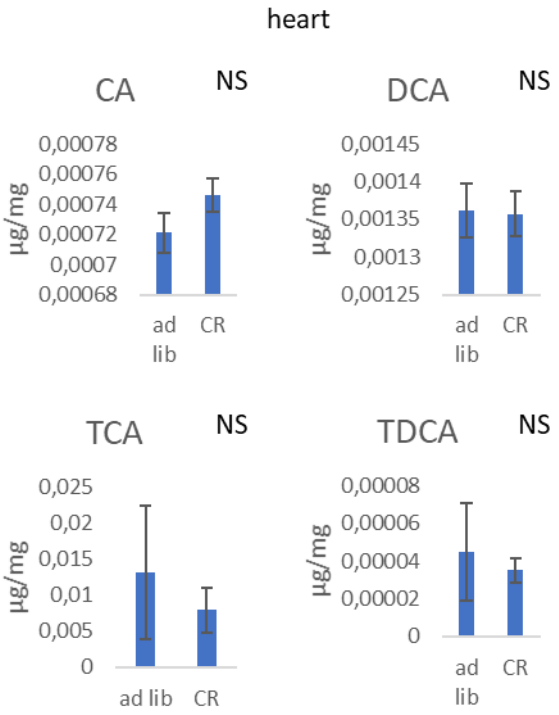

Figure 33: CA, DCA, TDCA and TCA concentrations measured in heart. No significances were measured.

In the heart, a trend towards higher concentrations of taurine-conjugated BAs in ad-lib mice can be observed (Figure 33).

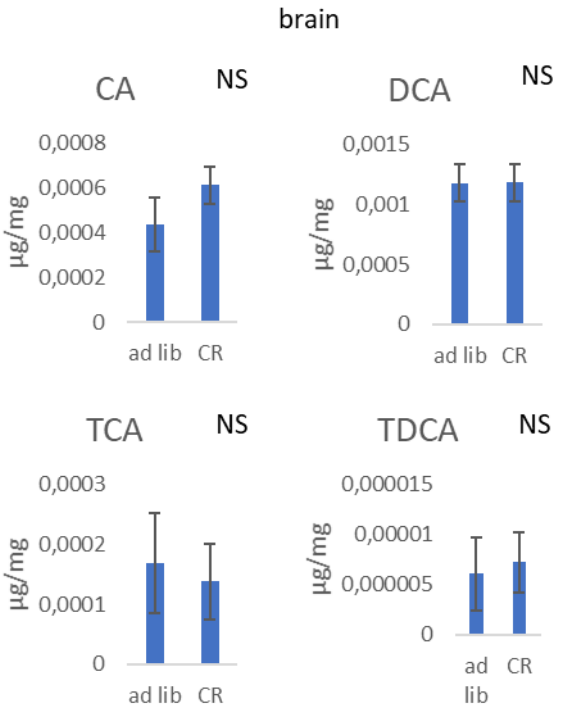

Figure 34: CA, DCA, TDCA and TCA concentrations measured in brain No significances were measured.

The only significance within all organs can be found for eWAT, where higher levels of CA are found in the CR group (Figure 32). The concentrations of brain and heart are neglectable (Figures 33, 34). Although there is virtually no significance the trend towards increased BA synthesis during CR can also be observed in most of the organs here. Especially in the kidney and spleen, substantial quantities of BA are measurable (Figures 29, 30).

4 Discussion

4.1 Bedding and fibre content

As seen in Gregor et al. bedding could affect the gut microbiota profile as well as faecal metabolites when mice are eating their bedding. When mice are fasting, they tend to eat more of the bedding (107).

It is well known among nutritionists that fibre binds BAs which results in an increase in BA excretion (via faeces). The extent of the binding capacity depends on the type of fibre and the substrate. This binding effect is one mechanism to lower cholesterol concentrations and promote BA excretion (115).

As seen in experiment I (bedding influence) fibre has an impact on BAs. The biggest rise of BA levels in mice's ileum and plasma is reached with the bedding cellulose in the CR group. This would indicate that cellulose has the worst binding affinity to BAs since BAs are not excreted more than usual when binding to fibre (107). In faeces, cellulose ad-lib had the biggest impact, this could mean that during fasting periods BAs are saved in the circulation (ileum/plasma/liver) and not excreted more via faeces due to fibre binding.

4.2 Microbiota influence

Throughout Experiment II the influence of the microbiome on BAs was investigated through different experiments with antibiotics and faecal transplants.

Since gut bacteria are responsible for the conversion from primary BAs into secondary BAs (53), it can be expected, that mice gavaged with antibiotics have no levels of DCA and TDCA in the ileum. This assumption has been proven. Since gut bacteria influence the BA pool (68), differences in the AT group are logical. If comparing AT-CR with CR itself, BA concentrations are higher in the CR group. Hence, the presence of the microbiota is important for the higher synthesis of BAs. One exception is taurine-conjugated primary BAs, which are also really high in the AT-CR group. Possibly not all bacteria were killed or CR itself has little impact on taurine-conjugated BAs. In the ileum, CR leads to higher BA concentrations compared to FT-CR. Even more interesting, especially in the ileum, FT ad-lib leads to higher BA concentrations

than FT-CR does. This is so astonishing since ad-lib itself always leads to lower BA levels than CR. Thus, one would expect lower BA concentrations if mice were gavaged FT ad-lib microbiome. Maybe the altered microbiome leads to such results.

Since CR itself has shown the biggest impact on BAs, compared to the FT and AT groups, it can be stated that the microbiome is responsible for changes in the BA pool size.

4.3 Transporters, receptors and transcription factors

4.3.1 FXR and TGR5

In the liver, hardly any differences in the FXR expression were detected, whereas in the ileum the highest expression is found in the CR group. Since FXR is involved in many BA metabolic processes such as absorption, hepatic uptake as well as the regulation of the feedback loop (54), it should be clear that higher BA levels should be attributable to a higher expression of FXR. The fact that no differences in the liver can be found, could be explained by the assumption that the microbiota only affects FXR in the ileum, which was described by Fiorucci et al. (68). Moreover, it has been shown, that the presence of the microbiota impacts the BA pool as it regulates FXR and diminish the BA pool (68). This probably explains controversial results, regarding the AT group.

Studies proved, that CR leads to a greater immune capacity (3), which is regulated by the receptors FXR and TRG5 (55). Hence, CR could evoke higher expression levels of FXR and TGR5, however, for TGR5 the results are less clear than for FXR.

4.3.2 FGF15 and FGF21

FGF15 in jejunum showed higher levels in the ad-lib group which could indicate that, under CR, the mechanism behind repressing or inhibiting CYP7A1 are less active. During ad-lib, more FGF15 is expressed. Thus, increased inhibition of CYP7A1 results in less BA synthesis. Whereas during CR a lower FGF15 expression is measured, hence less inhibition of CYP7A1 and therefore a greater BA synthesis is possible. As seen in Gregor et al. CYP7A1 is upregulated in the liver during CR (51). This again supports the greater synthesis of BAs during CR.

FGF21 is especially activated by prolonged fasting (71), therefore CR can probably cause the same effect. In this thesis, FGF21 was higher expressed during CR, FT-CR and AT-CR which supports the prolonged fasting effect. Interestingly, AT-CR has the highest expression. Thus, CR itself, without microbiota has the biggest impact. Whether this is just a coincidence or not, further measurements are needed.

4.3.3 CYP8B1

During feeding, CYP8B1 gets inhibited. Interestingly during restricted feeding, CYP8B1 is downregulated as well (89). Therefore, the comparison between ad-lib and CR is important. Ad-lib fed mice have slightly higher expression levels, additionally, also the AT ad-lib group show higher levels. Thus, CR has a bigger downregulating effect on CYP8B1 expression. CR leads to higher levels of BAs; thus, one would assume more CYP8B1 would be required for CA synthesis. Since CR leads to a downregulation of CYP8B1, less CA should be produced. Lower CA levels are attributed to many health benefits and mice lacking CYP8B1 have been associated with insulin sensitivity and higher glucose tolerance as well as diabetes and metabolic syndrome management (90). Hence health benefits of CR can be supported. However, in this thesis CR resulted in the highest concentration of CA.

4.3.4 IBAT

The ileal apical sodium dependent bile acid cotransporter (IBAT) was measured within two different settings, one within the bedding group as well as within the taurine supplemented diet group**.** In both groups, the expression results in controversial directions. ON fasting seems to lead to an increased expression, however, CR does not seem to have such an increasing effect. Comparing ad-lib with CR, IBAT show higher levels within ad-lib, or no clear differences at all. These results are really interesting since IBAT is responsible for the initial uptake of BAs and supports uptake into the EC. Mice under CR have higher BA synthesis and probably a higher circulation of BAs to compensate for deficiencies (53). This would assume higher expression levels of IBAT for the uptake of the higher amount of BAs. These findings make clear, that further research is needed in the field of EC, IBAT, BAs and CR.

4.3.5 PXR

PXR should be activated mostly through high BA levels, as it functions as a regulator for toxic BAs (74,78). PXR shows the highest expression during CR which is supported by the assumption that higher BA levels are circulating in mice organisms during CR. However, if treated with AT or FT, ad-lib leads to higher expression. After AT application no secondary BAs are produced, thus, less toxic BAs should be circulating in the organism. This fact makes the result of PXR in the AT and FT groups even more astonishing. Therefore, more research is needed.

4.3.6 LXRα, LXRβ, ABCA1, ABCG1 and SREBP1c

Since LXR receptors protect against a cholesterol overload (78) and LXR and the ATP cassette transporter are both parts of the reverse cholesterol transport (79), higher cholesterol levels would probably activate LXRα/β, ABCA1 and ABCG1, for cholesterol efflux and an increase in the fatty acid synthesis by upregulating SREBP1c. As reported in Fernández-Alvarez et al., SREBP1c is affected by the nutritional status and should be downregulated during fasting (81). Moreover, BAs are indirectly affecting SPREB1c by inhibiting LXR, followed by downregulating SREBP1c (80).

During CR more BAs are circulating in the liver and ileum, therefore LXR receptors and SREBP1c should get downregulated. However, during CR higher expressions of SRPEB1c and LXRβ are found. Additionally, also the cassette transporters are higher expressed. On the other hand, in all five genes (LXRα/β, ABCGA1, ABCG1, SREBP1c) AT-ad lib leads to the highest expression.

4.3.7 Ostα/β

Taking a look at the efflux transporters, the results are controversial. They are important for the export of BAs across the basolateral membrane (54), thus, higher BA synthesis, and higher turnover, should correlate with the transporters. However, ad-lib groups tend to have a greater impact, but without statistical significance.

4.4 Taurine diet content

Taurine is an organic sulfonic acid derivate, which mammals metabolise from the amino acids methionine and cysteine. Cysteine and methionine are included in proteins and are taken up through dietary protein (116). Mice with standard chow can metabolise taurine from cysteine and methionine since both amino acids are part of the standard chow. Within the LTD, neither taurine nor cysteine nor methionine are added to the food. Therefore, mice cannot synthesise taurine in their bodies. Concerning restricted feeding, taurine has already been suggested as a supplement for mimicking the CR effect (51). Hence, a comparison between the groups CR and taurine supplements is interesting to look at.

Interestingly, a CR-LTD has the biggest impact among the taurine supplementations on BAs in ileum and liver. This suggests that higher levels of taurine in mice organisms cannot increase BA synthesis if mice were calorically restricted. On the other hand, if comparing ad-lib with taurine-rich control groups (Tau, CTRL NTD), the taurine diets show higher BA concentrations in ileum than ad-lib does. Thus, if neither CR nor fasting are carried out, taurine supplementation in mice's drinking water is helpful for an increase of BAs and mimicking the BA-dependent fasting effect. CR itself has the highest BA levels in liver, therefore a diet with methionine and cysteine, but without taurine supplementation in CR mice has shown the highest BA concentration. Moreover, BAs are saved again in the mice's organisms during fasting periods, also if taurine is supplemented to the diet. In the ileum, CR does not influence BA increase much, taurine diets, however, especially with CR, showed the best BA increase. Therefore, no taurine or just only a small amount of it increased BAs even more.

4.5 CR and BAs

As described in Fu et al., CR does not affect BAs in the liver (50) which cannot be stated based on this thesis. CR has an impact on BAs in several tissues, especially in the ileum, liver and plasma but also in the spleen, kidney, heart and eWAT, where an increase in the BA concentration could be shown. Moreover, Fu et al. mentioned that unconjugated BAs in the small intestine also were not affected by CR (50). The results in this thesis again show the opposite.

Concerning faeces, the ad-lib application is responsible for the highest BA levels, which indicates, that during ad-lib more BAs are excreted. Whereas during fasting periods FGF15 and CYP8B1 are less clearly expressed, thus more BAs are synthesised and kept in the EC. A higher turnover of BAs and less excretion also means more efficient lipid- and lipid-vitamin absorption during CR. This process suggests that under restriction periods the organism tries to synthesise more BAs and do not excrete them immediately, hence saving them for lipid and vitamin absorption. This assumption could be true due to the function of insulin. Lower insulin levels occurring during long fasting periods are attributed to an upregulation of CYP7A1 (54), thus more BAs are synthesised.

4.6 KDs, IF, and FMD

The results of IF and FMD show that further forms of fasting have an impact on BAs as well. Interestingly, taurine-conjugated BAs are stronger affected by IF, whereas taurine-unconjugated BAs (DCA and CDCA) are more affected by FMD. To find out whether this is just a mere coincidence or not, further measurements should be carried out to support this assumption.

The KD control diet is rich in starch and protein and leads to the highest rise in BAs. Since TCA is expected to rise during a fat-rich diet (97), it is interesting to have a look at this specific BA as well. During the KD application, the control group always has the biggest impact on BAs, however, for TCA in the groups KD LCT and KD LCT/MCT slightly higher concentrations than in other BAs have been measured. However, also CA had slightly higher concentrations during KD treatment. Therefore, the question emerges if the rise of TCA can be attributed to the higher fat content of KD LCT and KD LCT/MCT. The assumption that a high-fat diet leads to higher BA excretion, cannot be verified, as BAs were only measured in the ileum and not in faeces.

As described in Li et al. (105), primary BA synthesis is downregulated during KD. This cannot be proven in this thesis. UDCA, CDCA, and TUDCA have lower concentrations, however, TCA and CA concentrations are quite high compared to the secondary BAs TLCA, TDCA, and DCA. Assumably, the effect of TCA could be attributed to the high-fat content. TDCA and CA have shown decreased concentrations in colitis mice in Li et al. (105). Therefore, CA in healthy

KD mice possibly does not evoke the same decreasing CA effect as in colitis mice. The LCT/MCT KD had a greater impact on BAs than the LCT KD. Thus, MCT possibly upregulates the BA synthesis. Since the control group leads to the highest BA concentrations, a diet rich in starch and protein possibly affects BA concentrations in the ileum.

4.7 Further measurements

Since some of the measurements led to controversial and unclear results, it could be recommendable to consider further experiments.

MCA, the most important BA in mice, has never been part of this measurement and thesis, hence, measuring MCA and comparing it to other primary BAs could be helpful.

Since there is the assumption that a high-fat diet leads to increased excretion of secondary BAs (58), it would be useful to measure the ketogenic diets in faeces as well, with a focus on secondary BAs.

Besides BAs, some transporters, receptors and transcriptional factors, involved in BA metabolism, should be measured again or under other conditions. Especially on FXR and TGR5 more focus is needed.

As the complete thesis focuses on mice's BA metabolism and BA changes in mice, more human studies in this field would be important and recommendable. Transferring animal study results to humans' metabolism is not always possible and due to differences in the BA metabolism further investigation on BAs and CR should be performed.

4.8 Limitations throughout the thesis

It has been tried to measure LCA, a secondary BA, in the first LCMS measurements and experiments. Unfortunately, the peak detection and the resulted concentrations were controversial and unclear, therefore this BA was eliminated from further measurements and not mentioned in this thesis. However, since DCA is the only measured secondary BA within this

thesis, LCA would have been important. As LCA is described as the most toxic BA, missing results have an even worse effect. In addition to LCA, DHCA was not measurable in the liver.

Moreover, peak detection was sometimes really complicated due to impurities and nitrogen loss during the LCMS measurement. Thus, correct interpretation was difficult in some cases.

5 Conclusion

During CR, it has been proven, that mice have a bigger BA pool size than ad-lib mice have. This effect can be supported by the transporters, receptors and TFs involved in the metabolism. FGF15 was less expressed during CR, hence, less repressing of CYP7A1 is possible. CYP7A1 and FXR are upregulated which enable greater BA synthesis. PXR, a toxic BA regulator, activated via high levels of BAs, is higher expressed during CR. FGF21, activated through fasting, shows higher expression during CR. Interestingly, Ostα/β and IBAT, responsible for transporting BAs, are not stronger induced during CR. Receptors, responsible for the cholesterol efflux, are partly more highly expressed during ad-lib.

Besides fasting, the content of the diet itself also affects BAs. Fibres, well known for binding BAs, do not lead to higher BA levels in faeces during CR. Although mice are consuming more (fibre-rich) bedding during CR, the BA excretion is still higher in the ad-lib group. There is also a difference between the type of fibres. Regarding bedding, cellulose leads to the biggest BA increase in ileum compared to wood and corncob. Summing up, CR and fibre-rich diets lead to higher BA concentrations in the ileum, however, they are not excreted to a higher extent.

Taurine is metabolised from the amino acids cysteine and methionine. If both amino acids are not part of the diet, no taurine is synthesised in mice. Taurine supplements are already known for mimicking a CR effect (51). If taurine is added to mice's drinking water, more BAs are produced and circulate in the EC of ad-lib mice. However, a CR diet without taurine, methionine and cysteine enhances synthesis even more. In the liver, CR itself containing methionine and cysteine in standard chow increases the BA pool most, whereas in the ileum especially the taurine diets with CR can enlarge BA concentration. The highest BA level was measured within the CR-LTD diet. Supplemented taurine without CR can help to maintain the higher BA synthesis fasting effect but CR itself, without taurine, still enhances BA levels more in ileum.

Regarding the effect of the microbiome, AT-CR and FT-CR do not show the same effect as CR does. Thus, CR itself cannot increase BA levels in the same way as with gut bacteria. It has been confirmed, that changes in the BA metabolism are dependent on the microbiome. Furthermore, no secondary BAs were produced due to gut bacteria depletion. Interestingly, FT-CR does not have the same effect as CR.

Apart from CR, other fasting types are able to affect BA concentrations. IF, as well as FMD, can increase BA's pool. Taurine-conjugated BAs are more affected by IF, whereas taurine-unconjugated BAs show higher concentrations under FMD. Concerning ketogenic diets, the assumption that a fat-rich diet leads to higher BA levels, cannot be verified. Since the control group shows the highest BA concentrations, a diet rich in starch and protein, probably will also affect BAs.

To put it into a nutshell, during fasting periods, such as CR, IF and FMD, mice synthesise more BAs and do not excrete them via faeces and save them in the enterohepatic circulation. Hence fat and fat-soluble vitamin absorptions are likely supported. Nevertheless, human data regarding CR is still rare. That is why more investigation in this field is needed to draw clear conclusions regarding BAs in humans.

References

1. Most J, Tosti V, Redman LM, Fontana L, Biomedical P, Rouge B, Science N, Sciences E, Avanzate B. Calorie restriction in humans: an update. 2018;36–45.

2. Libary of Congress [Internet]. [cited 2022 Sep 11]. Available from: https://id.loc.gov/authorities/names/n79063567.html

3. Speakman JR, Mitchell SE. Caloric restriction. Mol Aspects Med [Internet]. Elsevier Ltd; 2011;32:159–221. Available from: http://dx.doi.org/10.1016/j.mam.2011.07.001

4. Osborne TB, Mendel LB, Ferry EL. THE EFFECT OF RETARDATION OF GROWTH UPON THE BREEDING PERIOD AND DURATION OF LIFE OF RATS. Science [Internet]. American Association for the Advancement of Science; 1917 [cited 2022 Jul 2];45:294–5. Available from: https://www.science.org/doi/10.1126/science.45.1160.294

5. McCay CM, Crowell MF, Maynard LA. The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. Nutrition [Internet]. 1989;5:155–71; discussion 172. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2520283

6. Weindruch R. The retardation of aging by caloric restriction: studies in rodents and primates. Toxicol Pathol [Internet]. 1996;24:742–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8994305

7. Forster MJ, Morris P, Sohal RS. Genotype and age influence the effect of caloric intake on mortality in mice. FASEB J [Internet]. 2003;17:690–2. Available from: https://onlinelibrary.wiley.com/doi/10.1096/fj.02-0533fje

8. Longo VD, Fontana L. Calorie restriction and cancer prevention: metabolic and molecular mechanisms. Trends Pharmacol Sci [Internet]. 2010;31:89–98. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0165614709002028

9. Dirx MJ, Zeegers MP, Dagnelie PC, van den Bogaard T, van den Brandt PA. Energy restriction and the risk of spontaneous mammary tumors in mice: A meta-analysis. Int J Cancer [Internet]. 2003;106:766–70. Available from: https://onlinelibrary.wiley.com/doi/10.1002/ijc.11277

10. Kolaja KL, Bunting KA, Klaunig JE. Inhibition of tumor promotion and hepatocellular growth by dietary restriction in mice. Carcinogenesis [Internet]. 1996;17:1657–64. Available from: https://academic.oup.com/carcin/article/17/8/1657/2475911

11. Stewart JW, Koehler K, Jackson W, Hawley J, Wang W, Au A, Myers R, Birt DF. Prevention of mouse skin tumor promotion by dietary energy restriction requires an intact adrenal gland and glucocorticoid supplementation restores inhibition. Carcinogenesis [Internet]. 2005;26:1077–84. Available from: http://academic.oup.com/carcin/article/26/6/1077/2390855/Prevention-of-mouse-skin-tumor-promotion-by

12. Wheatley KE, Williams EA, Smith NCP, Dillard A, Park EY, Nunez NP, Hursting SD, Lane MA. Low-Carbohydrate Diet Versus Caloric Restriction: Effects on Weight Loss, Hormones, and Colon Tumor Growth in Obese Mice. Nutr Cancer [Internet]. 2007;60:61–8. Available from: http://www.tandfonline.com/doi/abs/10.1080/01635580701510150

13. Spady TJ, Lemus-Wilson AM, Pennington KL, Blackwood DJ, Paschall TM, Birt DF, McComb RD, Shull JD. Dietary energy restriction abolishes development of prolactin-producing pituitary tumors in Fischer 344 rats treated with 17beta-estradiol. Mol Carcinog [Internet]. 1998;23:86–95. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9808162

14. Zhu Z, Jiang W, Thompson HJ. An experimental paradigm for studying the cellular and molecular mechanisms of cancer inhibition by energy restriction. Mol Carcinog [Internet]. 2002;35:51–6. Available from: https://onlinelibrary.wiley.com/doi/10.1002/mc.10073

15. Lakatta EG, Levy D. Arterial and Cardiac Aging: Major Shareholders in Cardiovascular Disease Enterprises. Circulation [Internet]. 2003;107:346–54. Available from: http://www.circulationaha.org

16. Lakatta EG, Levy D. Arterial and Cardiac Aging: Major Shareholders in Cardiovascular Disease Enterprises. Circulation [Internet]. 2003;107:139–46. Available from: http://www.circulationaha.org

17. Walford RL, Harris SB, Gunion MW. The calorically restricted low-fat nutrient-dense

diet in Biosphere 2 significantly lowers blood glucose, total leukocyte count, cholesterol, and blood pressure in humans. Proc Natl Acad Sci U S A. 1992;89:11533–7.

18. Fontana L, Meyer TE, Klein S, Holloszy JO. Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. Proc Natl Acad Sci U S A. 2004;101:6659–63.

19. Fontana L. Calorie restriction and cardiometabolic health. Eur J Cardiovasc Prev Rehabil [Internet]. 2008;15:3–9. Available from: https://academic.oup.com/eurjpc/article/15/1/3-9/5933033

20. Fontana L, Villareal DT, Weiss EP, Racette SB, Steger-May K, Klein S, Holloszy JO. Calorie restriction or exercise: effects on coronary heart disease risk factors. A randomized, controlled trial. Am J Physiol Metab [Internet]. 2007;293:E197–202. Available from: http://www.ajpendo.org

21. Dhahbi JM, Tsuchiya T, Kim H-J, Mote PL, Spindler SR. Gene expression and physiologic responses of the heart to the initiation and withdrawal of caloric restriction. J Gerontol A Biol Sci Med Sci [Internet]. 2006;61:218–31. Available from: https://academic.oup.com/biomedgerontology/article/61/3/218/550387

22. Walford RL, Liu RK, Gerbase-Delima M, Mathies M, Smith GS. Longterm dietary restriction and immune function in mice: response to sheep red blood cells and to mitogenic agents. Mech Ageing Dev [Internet]. Mech Ageing Dev; 1973 [cited 2022 Jul 5];2:447–54. Available from: https://pubmed.ncbi.nlm.nih.gov/4599309/

23. Lustig A, Weeraratna AT, Wood WW, Teichberg D, Bertak D, Carter A, Poosala S, Firman J, Becker KG, Zonderman AB, et al. Transcriptome analysis of age-, gender- and diet-associated changes in murine thymus. Cell Immunol [Internet]. Cell Immunol; 2007 [cited 2022 Jul 5];245:42–61. Available from: https://pubmed.ncbi.nlm.nih.gov/17499630/

24. Jolly CA. Recent Advances in Nutritional Sciences Dietary Restriction and Immune Function 1 [Internet]. J. Nutr. 2004. Available from: https://academic.oup.com/jn/article/134/8/1853/4688853

25. Abe T, Nakajima A, Satoh N, Ohkoshi M, Sakuragi S, Koizumi A. Suppression of

experimental autoimmune uveoretinitis by dietary calorie restriction. Jpn J Ophthalmol [Internet]. Jpn J Ophthalmol; 2001 [cited 2022 Jul 5];45:46–52. Available from: https://pubmed.ncbi.nlm.nih.gov/11163045/

26. Effros RB, Walford RL, Weindruch R, Mitcheltree C. Influences of Dietary Restriction on Immunity to Influenza in Aged Mice. J Gerontol [Internet]. J Gerontol; 1991 [cited 2022 Jul 5];46:B142–7. Available from: https://pubmed.ncbi.nlm.nih.gov/2071828/

27. Roecker EB, Kemnitz JW, Ershler WB, Weindruch R. Reduced immune responses in rhesus monkeys subjected to dietary restriction. J Gerontol A Biol Sci Med Sci [Internet]. 1996;51:B276-9. Available from: https://academic.oup.com/biomedgerontology/article/51A/4/B276/618114

28. Messaoudi I, Fischer M, Warner J, Park B, Mattison J, Ingram DK, Totonchy T, Mori M, Nikolich-Žugich J. Optimal window of caloric restriction onset limits its beneficial impact on T-cell senescence in primates. Aging Cell [Internet]. 2008;7:908–19. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1474-9726.2008.00440.x

29. MERRY BJ. Calorie Restriction and Age-Related Oxidative Stress. Ann N Y Acad Sci [Internet]. Ann N Y Acad Sci; 2006 [cited 2022 Jul 5];908:180–98. Available from: https://pubmed.ncbi.nlm.nih.gov/10911958/

30. Merry BJ. Oxidative stress and mitochondrial function with aging - the effects of calorie restriction. Aging Cell [Internet]. 2004;3:7–12. Available from: http://doi.wiley.com/10.1046/j.1474-9728.2003.00074.x

31. Cui J, Panse S, Falkner B. The role of adiponectin in metabolic and vascular disease: a review. Clin Nephrol [Internet]. 2011;75:26–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21176748

32. Qiao L, Lee B, Kinney B, Yoo H sun, Shao J. Energy intake and adiponectin gene expression. Am J Physiol Metab [Internet]. 2011;300:E809–16. Available from: http://www.ajpendo.org

33. Aspnes LE, Lee CM, Weindruch R, Chung SS, Roecker EB, Aiken JM. Caloric restriction reduces fiber loss and mitochondrial abnormalities in aged rat muscle. FASEB J [Internet]. 1997;11:573–81. Available from:

https://onlinelibrary.wiley.com/doi/abs/10.1096/fasebj.11.7.9212081

34. Colman RJ, Beasley TM, Allison DB, Weindruch R. Attenuation of sarcopenia by dietary restriction in rhesus monkeys. J Gerontol A Biol Sci Med Sci [Internet]. 2008;63:556–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18559628

35. Baek K, Barlow AA, Allen MR, Bloomfield SA. Food restriction and simulated microgravity: effects on bone and serum leptin. J Appl Physiol [Internet]. 2008;104:1086–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18276897

36. Kalu DN, Hardin RR, Cockerham R, Yu BP, Norling BK, Egan JW. Lifelong food restriction prevents senile osteopenia and hyperparathyroidism in F344 rats. Mech Ageing Dev [Internet]. 1984;26:103–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6748753

37. Kemnitz JW. Calorie restriction and aging in nonhuman primates. ILAR J [Internet]. 2011;52:66–77. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21411859

38. Massaro D, Massaro GD, Baras A, Hoffman EP, Clerch LB. Calorie-related rapid onset of alveolar loss, regeneration, and changes in mouse lung gene expression. Am J Physiol Lung Cell Mol Physiol [Internet]. 2004;286:L896-906. Available from: http://www.ajplung.orgl896

39. Brecchia G, Bonanno A, Galeati G, Federici C, Maranesi M, Gobbetti A, Zerani M, Boiti C. Hormonal and metabolic adaptation to fasting: effects on the hypothalamic-pituitary-ovarian axis and reproductive performance of rabbit does. Domest Anim Endocrinol [Internet]. 2006;31:105–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16219443

40. Caprio M, Fabbrini E, Isidori AM, Aversa A, Fabbri A. Leptin in reproduction. Trends Endocrinol Metab [Internet]. 2001;12:65–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11167124

41. BALL ZB, BARNES RH, VISSCHER MB. The effects of dietary caloric restriction on maturity and senescence, with particular reference to fertility and longevity. Am J Physiol [Internet]. 1947;150:511–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20265851

42. Osborne TB, Mendel LB, Ferry EL. THE EFFECT OF RETARDATION OF GROWTH UPON THE BREEDING PERIOD AND DURATION OF LIFE OF RATS. Science [Internet]. 1917;45:294–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17760202

43. Kalm LM, Semba RD. They starved so that others be better fed: Remembering ancel keys and the Minnesota experiment. J Nutr. 2005;135:1347–52.

44. CR Society International [Internet]. [cited 2021 Nov 9]. Available from: https://www.crsociety.org/index.html/

45. Li T, Matozel M, Boehme S, Kong B, Nilsson L-M, Guo G, Ellis E, Chiang JYL. Overexpression of Cholesterol 7a-Hydroxylase Promotes Hepatic Bile Acid Synthesis and Secretion and Maintains Cholesterol Homeostasis. 2010;

46. Park W-H, Pak YK. Insulin-dependent suppression of cholesterol 7α-hydroxlase is a possible link between glucose and cholesterol metabolisms. Exp Mol Med [Internet]. 2011;43:571–9. Available from: http://dx.doi.org/10.3858/emm.2011.43.10.064

47. Pasiakos SM, Caruso CM, Kellogg MD, Kramer FM, Lieberman HR. Appetite and endocrine regulators of energy balance after 2 days of energy restriction: insulin, leptin, ghrelin, and DHEA-S. Obesity (Silver Spring) [Internet]. 2011;19:1124–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21212768

48. De Fabiani E, Mitro N, Gilardi F, Caruso D, Galli G, Crestani M. Coordinated control of cholesterol catabolism to bile acids and of gluconeogenesis via a novel mechanism of transcription regulation linked to the fasted-to-fed cycle. J Biol Chem [Internet]. 2003;278:39124–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12865425

49. Ponugoti B, Fang S, Kemper JK. Functional interaction of hepatic nuclear factor-4 and peroxisome proliferator-activated receptor-gamma coactivator 1alpha in CYP7A1 regulation is inhibited by a key lipogenic activator, sterol regulatory element-binding protein-1c. Mol Endocrinol [Internet]. 2007;21:2698–712. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17636037

50. Fu ZD, Klaassen CD. Increased Bile Acids in Enterohepatic Circulation by Short-term Calorie Restriction in Male Mice. Toxicol Appl Pharmacol. 2013;273(3):680–90.

51. Gregor A, Pignitter M, Fahrngruber C, Bayer S, Somoza V, König J, Duszka K. Caloric restriction increases levels of taurine in the intestine and stimulates taurine uptake by conjugation to glutathione. J Nutr Biochem. 2021;96.

52. Chen X, Lou G, Meng Z, Huang W. TGR5: a novel target for weight maintenance and glucose metabolism. Exp Diabetes Res [Internet]. 2011;2011:853501. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21754919

53. Xie C, Huang W, Young RL, Jones KL, Horowitz M, Rayner CK, Wu T. Role of bile acids in the regulation of food intake, and their dysregulation in metabolic disease. Nutrients. 2021;13:1–16.

54. Chiang JYL. Bile acid metabolism and signaling. Compr Physiol. 2013;3:1191–212.

55. Fiorucci S, Biagioli M, Zampella A, Distrutti E. Bile acids activated receptors regulate innate immunity. Front Immunol. 2018;9:1–17.

56. Ferdinandusse S, Houten SM. Peroxisomes and bile acid biosynthesis. Biochim Biophys Acta - Mol Cell Res. 2006;1763:1427–40.

57. Russell DW. The enzymes, regulation, and genetics of bile acid synthesis. Annu Rev Biochem [Internet]. 2003;72:137–74. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12543708

58. Di Ciaula A, Garruti G, Baccetto RL, Molina-Molina E, Bonfrate L, Wang DQH, Portincasa P. Bile acid physiology. Ann Hepatol [Internet]. Elsevier; 2017;16:s4–14. Available from: http://dx.doi.org/10.5604/01.3001.0010.5493

59. Fu ZD, Cui JY, Klaassen CD. The role of sirt1 in bile acid regulation during calorie restriction in mice. PLoS One. 2015;10:1–15.

60. Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, Luo G, Jones SA, Goodwin B, Richardson JA, et al. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. Cell Metab. 2005;2:217–25.

61. Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T, et al. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature [Internet]. 2006;439:484–9.

Available from: http://www.nature.com/articles/nature04330

62. Yokota A, Fukiya S, Islam KBMS, Ooka T, Ogura Y, Hayashi T, Hagio M, Ishizuka S. Is bile acid a determinant of the gut microbiota on a high-fat diet? Gut Microbes [Internet]. 2012;3:455–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22825495

63. Cariou B, Chetiveaux M, Zaïr Y, Pouteau E, Disse E, Guyomarc'h-Delasalle B, Laville M, Krempf M. Fasting plasma chenodeoxycholic acid and cholic acid concentrations are inversely correlated with insulin sensitivity in adults. Nutr Metab (Lond) [Internet]. BioMed Central Ltd.; 2011 [cited 2022 Jul 12];8:48. Available from: https://nutritionandmetabolism.biomedcentral.com/articles/10.1186/1743-7075-8-48

64. Straniero S, Rosqvist F, Edholm D, Ahlström H, Kullberg J, Sundbom M, Risérus U, Rudling M. Acute caloric restriction counteracts hepatic bile acid and cholesterol deficiency in morbid obesity. J Intern Med [Internet]. 2017;281:507–17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28261926

65. Prinz P, Hofmann T, Ahnis A, Elbelt U, Goebel-Stengel M, Klapp BF, Rose M, Stengel A. Plasma bile acids show a positive correlation with body mass index and are negatively associated with cognitive restraint of eating in obese patients. Front Neurosci [Internet]. 2015;9:199. Available from: www.frontiersin.org

66. Tu H, Okamoto AY, Shan B. FXR, a bile acid receptor and biological sensor. Trends Cardiovasc Med [Internet]. 2000;10:30–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11150726

67. Frankenberg T, Rao A, Chen F, Haywood J, Shneider BL, Dawson PA. Regulation of the mouse organic solute transporter alpha-beta, Ostalpha-Ostbeta, by bile acids. Am J Physiol Gastrointest Liver Physiol [Internet]. 2006;290:G912-22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16357058

68. Fiorucci S, Distrutti E. Bile Acid-Activated Receptors, Intestinal Microbiota, and the Treatment of Metabolic Disorders. Trends Mol Med [Internet]. Elsevier Ltd; 2015;21:702–14. Available from: http://dx.doi.org/10.1016/j.molmed.2015.09.001

69. Dawson PA. Role of the intestinal bile acid transporters in bile acid and drug disposition.

Handbook of Experimental Pharmacology. 2011. 169–203 p.

70. Ballatori N. Biology of a novel organic solute and steroid transporter, OSTalpha-OSTbeta. Exp Biol Med (Maywood) [Internet]. 2005;230:689–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16246895

71. Kliewer SA, Mangelsdorf DJ. Fibroblast growth factor 21: From pharmacology to physiology. Am J Clin Nutr. 2010;91:254–7.

72. Grabacka M, Pierzchalska M, Dean M, Reiss K. Regulation of ketone body metabolism and the role of PPARα. Int J Mol Sci. 2016;17.

73. Geach T. Neuroendocrinology: FGF21 influences a “sweet tooth” in mice. Nat Rev Endocrinol. Nature Publishing Group; 2016;12:123.

74. Kliewer SA, Goodwin B, Willson TM. The nuclear pregnane X receptor: A key regulator of xenobiotic metabolism. Endocr Rev. 2002;23:687–702.

75. Chiang JYL, Ferrell JM. Up to date on cholesterol 7 alpha-hydroxylase (CYP7A1) in bile acid synthesis. Liver Res [Internet]. 2020;4:47–63. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC8291349

76. Javitt NB. Cholestasis in rats induced by taurolithocholate. Nature [Internet]. Macmillan; 1966;210:1262–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/5967804

77. Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, et al. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc Natl Acad Sci U S A [Internet]. 2001;98:3369–74. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11248085

78. Li T, Chen W, Chiang JYL. PXR induces CYP27A1 and regulates cholesterol metabolism in the intestine. J Lipid Res [Internet]. Â© 2007 ASBMB. Currently published by Elsevier Inc; originally published by American Society for Biochemistry and Molecular Biology.; 2007;48:373–84. Available from: http://dx.doi.org/10.1194/jlr.M600282-JLR200

79. Gabbi C, Warner M, Gustafsson JÅ. Minireview: Liver X receptor β: emerging roles in

physiology and diseases. Mol Endocrinol. 2009;23:129–36.

80. Wanabe M, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, Heyman RA, Moore DD, Auwerx J. Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. J Clin Invest. 2004;113:1408–18.

81. Fernández-Alvarez A, Soledad Alvarez M, Gonzalez R, Cucarella C, Muntané J, Casado M. Human SREBP1c expression in liver is directly regulated by Peroxisome Proliferator-activated Receptor α (PPARα). J Biol Chem. 2011;286:21466–77.

82. Foufelle F, Ferré P. New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. Biochem J [Internet]. Portland Press Ltd; 2002 [cited 2022 Jul 13];366:377–91. Available from: /pmc/articles/PMC1222807/?report=abstract

83. Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ, et al. Role of LXRs in control of lipogenesis. Genes Dev [Internet]. 2000;14:2831–8. Available from: www.genesdev.org/cgi/doi/10.1101/

84. Bateson MC, Maclean D, Evans JR, Bouchier IA. Chenodeoxycholic acid therapy for hypertriglyceridaemia in men. Br J Clin Pharmacol [Internet]. 1978;5:249–54. Available from: http://www.ncbi.nlm.nih.gov/pubmed/656270

85. Angelin B, Einarsson K, Hellström K, Leijd B. Effects of cholestyramine and chenodeoxycholic acid on the metabolism of endogenous triglyceride in hyperlipoproteinemia. J Lipid Res [Internet]. 1978;19:1017–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/731123

86. Carulli N, Ponz de Leon M, Podda M, Zuin M, Strata A, Frigerio G, Digrisolo A. Chenodeoxycholic acid and ursodeoxycholic acid effects in endogenous hypertriglyceridemias. A controlled double-blind trial. J Clin Pharmacol [Internet]. 1981;21:436–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7031100

87. Gåfvels M, Olin M, Chowdhary BP, Raudsepp T, Andersson U, Persson B, Jansson M, Björkhem I, Eggertsen G. Structure and chromosomal assignment of the sterol 12α-hydroxylase gene (CYP8B1) in human and mouse: Eukaryotic cytochrome P-450 gene devoid of introns. Genomics. 1999;56:184–96.

88. Yang Y, Eggertsen G, Gåfvels M, Andersson U, Einarsson C, Björkhem I, Chiang JYL. Mechanisms of cholesterol and sterol regulatory element binding protein regulation of the sterol 12α-hydroxylase gene (CYP8B1). Biochem Biophys Res Commun. 2004;320:1204–10.

89. Pathak P, Li T, Chiang JYL. Retinoic acid-related orphan receptor α regulates diurnal rhythm and fasting induction of sterol 12α-hydroxylase in bile acid synthesis. J Biol Chem [Internet]. 2013 [cited 2022 Jul 14];288:37154–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24226095

90. Kaur A, Patankar J V., De Haan W, Ruddle P, Wijesekara N, Groen AK, Verchere CB, Singaraja RR, Hayden MR. Loss of Cyp8b1 improves glucose homeostasis by increasing GLP-1. Diabetes. 2015;64:1168–79.

91. Thakare R, Alamoudi JA, Gautam N, Rodrigues AD, Alnouti Y. Species differences in bile acids I. Plasma and urine bile acid composition. J Appl Toxicol. 2018;38:1323–35.

92. Straniero S, Laskar A, Savva C, Härdfeldt J, Angelin B, Rudling M. Of mice and men: Murine bile acids explain species differences in the regulation of bile acid and cholesterol metabolism. J Lipid Res. 2020;61:480–91.

93. Patterson RE, Sears DD. Metabolic Effects of Intermittent Fasting. Annu Rev Nutr. 2017;37:371–93.

94. Longo VD, Mattson MP. Fasting: Molecular mechanisms and clinical applications. Cell Metab [Internet]. Elsevier Inc.; 2014;19:181–92. Available from: http://dx.doi.org/10.1016/j.cmet.2013.12.008

95. Brandhorst S, Choi IY, Wei M, Cheng CW, Sedrakyan S, Navarrete G, Dubeau L, Yap LP, Park R, Vinciguerra M, et al. A Periodic Diet that Mimics Fasting Promotes Multi-System Regeneration, Enhanced Cognitive Performance, and Healthspan. Cell Metab. Elsevier Inc.; 2015;22:86–99.

96. Cheng CW, Villani V, Buono R, Wei M, Kumar S, Yilmaz OH, Cohen P, Sneddon JB, Perin L, Longo VD. Fasting-Mimicking Diet Promotes Ngn3-Driven β-Cell Regeneration to Reverse Diabetes. Cell [Internet]. Elsevier; 2017;168:775-788.e12. Available from: http://dx.doi.org/10.1016/j.cell.2017.01.040

97. Zarnowska IM. Therapeutic use of the ketogenic diet in refractory epilepsy: What we know and what still needs to be learned. Nutrients. 2020;12:1–23.

98. Kossoff EH, Zupec-Kania BA, Auvin S, Ballaban-Gil KR, Christina Bergqvist AG, Blackford R, Buchhalter JR, Caraballo RH, Cross JH, Dahlin MG, et al. Optimal clinical management of children receiving dietary therapies for epilepsy: Updated recommendations of the International Ketogenic Diet Study Group. Epilepsia Open [Internet]. 2018;3:175–92. Available from: https://onlinelibrary.wiley.com/doi/10.1002/epi4.12225

99. Augustin K, Khabbush A, Williams S, Eaton S, Orford M, Cross JH, Heales SJR, Walker MC, Williams RSB. Mechanisms of action for the medium-chain triglyceride ketogenic diet in neurological and metabolic disorders. Lancet Neurol [Internet]. 2018;17:84–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29263011

100. Wlaź P, Socała K, Nieoczym D, Łuszczki JJ, Zarnowska I, Zarnowski T, Czuczwar SJ, Gasior M. Anticonvulsant profile of caprylic acid, a main constituent of the medium-chain triglyceride (MCT) ketogenic diet, in mice. Neuropharmacology [Internet]. 2012;62:1882–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22210332

101. Muzykewicz DA, Lyczkowski DA, Memon N, Conant KD, Pfeifer HH, Thiele EA. Efficacy, safety, and tolerability of the low glycemic index treatment in pediatric epilepsy. Epilepsia. 2009;50:1118–26.

102. Pfeifer HH, Thiele EA. Low-glycemic-index treatment: a liberalized ketogenic diet for treatment of intractable epilepsy. Neurology [Internet]. Wolters Kluwer Health, Inc. on behalf of the American Academy of Neurology; 2005 [cited 2022 Aug 15];65:1810–2. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16344529

103. Brynes AE, Adamson J, Dornhorst A, Frost GS. The beneficial effect of a diet with low glycaemic index on 24 h glucose profiles in healthy young people as assessed by continuous glucose monitoring. Br J Nutr [Internet]. 2005;93:179–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15788110

104. Yoshitsugu R, Kikuchi K, Iwaya H, Fujii N, Hori S, Lee DG, Ishizuka S. Alteration of bile acid metabolism by a high-fat diet is associated with plasma transaminase activities and glucose intolerance in rats. J Nutr Sci Vitaminol (Tokyo). 2019;65:45–51.

105. Li S, Zhuge A, Wang K, Lv L, Bian X, Yang L, Xia J, Jiang X, Wu W, Wang S, et al. Ketogenic diet aggravates colitis, impairs intestinal barrier and alters gut microbiota and metabolism in DSS-induced mice. Food Funct [Internet]. 2021;12:10210–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/34542110

106. Song HK, Hwang DY. Use of C57BL/6N mice on the variety of immunological researches. Lab Anim Res. 2017;33:119.

107. Gregor A, Fragner L, Trajanoski S, Li W, Sun X, Weckwerth W, König J, Duszka K. Cage bedding modifies metabolic and gut microbiota profiles in mouse studies applying dietary restriction. Sci Rep. 2020;10:1–13.

108. Wegner K, Just S, Gau L, Mueller H, Gérard P, Lepage P, Clavel T, Rohn S. Rapid analysis of bile acids in different biological matrices using LC-ESI-MS/MS for the investigation of bile acid transformation by mammalian gut bacteria. Anal Bioanal Chem [Internet]. Analytical and Bioanalytical Chemistry; 2017;409:1231–45. Available from: http://dx.doi.org/10.1007/s00216-016-0048-1

109. Dass C. Hyphenated Separation Techniques. Fundamentals of Contemporary Mass Spectrometry,. Hoboken, NJ, USA; 2007. p. 151–94.

110. Liu Y, Rong Z, Xiang D, Zhang C, Liu D. Detection technologies and metabolic profiling of bile acids: A comprehensive review. Lipids Health Dis. Lipids in Health and Disease; 2018;17:1–13.

111. Shimadzu Corporation. Fundamental Guide to Liquid Chromatography Mass Spectrometry (LCMS). 2019;62. Available from: https://www.shimadzu.eu.com/sites/shimadzu.seg/files/SEG/C10G-E065A_Shimadzu_Fundamental_Guide_to_LCMS.pdf

112. Sinha A, Mann M. Beginner ' s Guide A beginner ' s guide to RT- - Beginner ' s Guide. 2020;0:1–6. Available from: https://doi.org/10.1042/BIO20200034

113. Navarro E, Serrano-Heras G, Castaño MJ, Solera J. Real-time PCR detection chemistry. Clin Chim Acta [Internet]. Elsevier B.V.; 2015;439:231–50. Available from: http://dx.doi.org/10.1016/j.cca.2014.10.017

114. Pabinger S, Rödiger S, Kriegner A, Vierlinger K, Weinhäusel A. A survey of tools for the analysis of quantitative PCR (qPCR) data. Biomol Detect Quantif [Internet]. Elsevier GmbH; 2014;1:23–33. Available from: http://dx.doi.org/10.1016/j.bdq.2014.08.002

115. Kritchevsky D. Influence of dietary fiber on bile acid metabolism. Lipids. 1978;13:982–5.

116. Nakamura H, Yatsuki J, Ubuka T. Production of hypotaurine, taurine and sulfate in rats and mice injected with L-cysteinesulfinate. Amino Acids [Internet]. 2006;31:27–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16680400