Diplomarbeit

Does the Microbiome Affect Intestinal Steroidogenesis?

eingereicht von

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zur Erlangung des akademischen Grades

Doktor(in) der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt an der

Universitätsklinik für Innere Medizin

Klinische Abteilung für Gastroenterologie und Hepatologie

unter der Anleitung von

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Graz, 09.11.2020

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Graz, 09.11.2020 *Michaela Krainer eh*

Danksagungen

Ich möchte mich an dieser Stelle bei allen bedanken, die mir bei der Erstellung dieser Diplomarbeit geholfen haben.

Besonders bei Martin Wagner und Katrin Panzitt möchte ich mich bedanken, für die großartige Betreuung und Geduld.
Ein großes Danke geht an das ganze Laborteam, allen voran Judith sowie Dagmar und Silvia, für die Unterstützung bei den Experimenten und Hilfe bei jeglichen Fragen.

Schließlich möchte ich mich auch bei meinen Eltern bedanken, für die Unterstützung während meines gesamten Studiums.

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List of Abbreviations

KR	Kernrezeptor
CED	chronisch entzündliche Darmerkrankungen
FMT	fecal microbiota transplantation
WB	Western Blot
IF	immunofluorescence
NR	nuclear receptor
IBD	inflammatory bowel disease
GC	glucocorticoid
GR	glucocorticoid receptor
CRH	corticotropin releasing hormone
ACT	adrenocorticotropic hormone
HPA axis	hypothalamic-pituitary-adrenal axis
StAR	steroidogenic acute regulatory protein
IMM	inner mitochondrial membrane
SF-1	steroidogenic factor 1
CYP	cytochrome P450 heme-containing proteins
HSD	hydroxysteroid dehydrogenases
DHEA	dehydroepiandrosterone
LRH-1	liver receptor homolog-1
NTD	N-terminal domain
AF-1	activation function-1 region
DBD	DNA binding domain
LBD	ligand binding domain
AF-2	activation function-2 region
RXR	retinoid-X-receptor
SHP	small heterodimer partner
FXR	farnesoid-X-receptor
MBF1	multiprotein bridging factor
DLPC	dilauryol-phosphatidyl-choline
APC	adenomatous polyposis coli
FAP	familial adenomatous polyposis
AOM	azoxymethane
TNBS	2,4,6-trinitrobenzene sulfonic acid
DSS	dextran sodium sulfate
CD	Crohn's Disease
UC	Ulcerative Colitis
PPARγ	peroxisome proliferator-activated receptor γ
PC	phosphatidylcholine
TJB	tight junction barrier
TNF-α	tumor necrosis factor-α
MLCK	myosin light chain kinase
TBP	TATA-box binding protein
FGF15	fibroblast growth factor 15
GCK	glucokinase
APOA4	apolipoprotein A-IV

Figures and Tables

Zusammenfassung

Einleitung: Der Darm ist eine Quelle lokaler Glukokortikoidsynthese, welche relevant für die Erhaltung intestinaler Immunhomöostase ist. Enzyme, die an der intestinalen Steroidsynthese beteiligt sind, werden durch den Kernrezeptor (KR) LRH-1 reguliert. Der KR LRH-1 sowie die Enzyme der Steroidsynthese sind in entzündeten Bereichen des Kolons von Personen mit chronisch entzündlichen Darmerkrankungen (CED) schwächer exprimiert als in gesundem Gewebe. Fecal microbiota transplantation (FMT) ist eine vielversprechende therapeutische Option für CED Patientinnen und Patienten mit Colitis Ulcerosa. Wir stellen daher die Hypothese auf, dass das Darmmikrobiom die lokale Steroidsynthese beeinflusst.

Methoden: Um den Einfluss des Mikrobioms auf die intestinale Steroidsynthese zu bestimmen, verglichen wir verschiedene Abschnitte des Darms (Jejunum, Ileum und Kolon) von keimfreien und konventionellen Mäusen (jeweils n=5). Wir fokussierten uns auf die Analyse der Schlüsselenzyme der intestinalen Steroidsynthese Cyp11a1, Cyp11b1 und Hsd3b, welche alle durch LRH-1 reguliert werden. Um Expressionslevels zu testen, führten wir RT-qPCRs, Western Blots (WB) und Immunfluoreszenzfärbungen (IF) durch.

Resultate: Am deutlichsten sah man die Unterschiede auf Proteinebene bei Cyp11a1 und Cyp11b1, welche eine signifikant erniedrigte Expression in keimfreien Mäusen zeigten. Auf mRNA Ebene zeigten sich die deutlichsten Veränderungen bei Hsd3b1 und Hsd3b3, welche im Kolon von keimfreien Mäusen signifikant niedriger exprimiert waren. Im Gegensatz dazu waren diese Enzyme im Ileum von keimfreien Mäusen höher exprimiert. In der IF zeigte sich eine reduzierte Expression von Cyp11a1 im Kolon von keimfreien Mäusen.

Diskussion: Das Darmmikrobiom hat signifikanten Einfluss auf die Expression der Enzyme der Steroidsynthese. Cyp11a1 und Cyp11b1 sind deutlich reduziert in keimfreien Mäusen. Die Rolle von LRH-1 in Zusammenhang mit diesen Veränderungen ist zurzeit noch unklar und kann in weiteren Untersuchungen (z.B. LRH-1 Chromatin Immunpräzipitation) analysiert werden.

Abstract

Introduction: The intestine is a source of locally active glucocorticoid generation, which is relevant for the maintenance of intestinal immune homeostasis. Steroidogenic enzymes in the intestine are regulated by the nuclear receptor (NR) LRH-1. The expression of both, steroidogenic enzymes as well as the regulating NR LRH-1 is reduced in inflamed parts of the colon of patients with inflammatory bowel disease (IBD). Fecal microbiota transplantation is a promising experimental therapeutic option for IBD patients with ulcerative colitis. We therefore hypothesize, that the gut microbiome may affect local steroidogenesis.

Methods: To determine the effect of the microbiome on intestinal steroidogenesis, we compared different parts of the intestine (jejunum, ileum, and colon) of germfree and conventional mice (n=5 per group). We focused on the key steroidogenic enzymes Cyp11a1, Cyp11b1 and Hsd3b, which are all regulated by LRH-1. To test expression levels, we performed RT-qPCR, Western Blotting (WB) and immunofluorescence (IF) staining.

Results: Differences were most pronounced on protein levels for Cyp11a1 and Cyp11b1, which were significantly lower in germfree mice. On mRNA levels the most robust changes were detected for Hsd3b1 and Hsd3b3, which were expressed significantly less in the colon of germfree mice contrary to increased expression in the ileum of germfree mice. IF showed lower expression of Cyp11b1 in the colon of germfree mice.

Discussion: The presence of the gut microbiome significantly affects steroidogenic enzyme expression in the intestine. Cyp11a1 and Cyp11b1 are significantly less expressed in germfree mice. The role of LRH-1 in mediating these effects is currently not clear and further in-depth analysis (e.g. LRH-1 chromatin immunoprecipitations) is required.

1 Background

1.1 Adrenal Steroidogenesis

In humans and other mammals, the main synthesis sites for steroids are the adrenal glands and gonads. The main end products of adrenal steroidogenesis are glucocorticoids (GCs), mineralocorticoids, and androgens (Figure 1). The focus of this thesis lies on glucocorticoids and their contribution to gut health.

Beside the adrenal glands many other tissues including liver, lungs and intestines have the capacity to synthesize steroids de novo or to activate inactive circulating precursors. Peripherally produced steroids do not add to systemic levels but act locally (1). Mammals produce the glucocorticoids cortisol and corticosterone in varying ratios. The predominant active glucocorticoid in humans is cortisol, in rodents it is corticosterone (2).

1.1.1 Overview on the Effects of Systemic Glucocorticoids Produced by Adrenal Glands

Glucocorticoids help the body deal with physical and emotional stress (3). They provide energy in the form of glucose and fatty acids by promoting gluconeogenesis and lipolysis and regulate immune and inflammatory processes (4). They also affect the sensitivity of tissue to catecholamines, which leads to raised blood pressure. Furthermore, they have effects on electrolyte and water balance, bone density, cell growth, neuronal plasticity, and neurodegeneration. Mood and behavior can be influenced by glucocorticoid levels as well as food intake and pain perception (2).

These effects are wanted and necessary for preserving the organism under the influence of stressors. However, prolonged high levels of glucocorticoids, e.g. due to long lasting stress, pathological endogenous synthesis, or prolonged administration of glucocorticoids, have a negative impact on the body. Consequences can be obesity, hypertension, immunodeficiency, muscle loss, diabetes, hypercholesterolemia, osteoporosis, and depression (2). Glucocorticoids are ligands for the glucocorticoid receptor (GR). GR is a nuclear receptor and transcription factor that is expressed ubiquitously and resides in the cytoplasm in its inactive state (5-7). When GCs binds to GR, it is translocated to the nucleus, where GR regulates transcription for its target genes (5-7).

1.1.2 Regulation of Adrenal Steroidogenesis

Unlike many other hormones, steroids are not stored in significant amounts. Thus, in the case of stress triggers and the sudden need of steroids, they must be rapidly synthesized (8). Synthesis in the adrenal glands is regulated by the hypothalamic-pituitary-adrenal axis (HPA axis). With a circadian rhythm dictated by the light-dark cycle and corticotropin releasing hormone (CRH) being released by the hypothalamus, adrenocorticotropic hormone (ACTH) is released by the pituitary gland in approximately 20 pulses in 24 hours (8). Highest levels of basal ACTH are reached before 7 am, lowest levels between 11 pm and 3 am (9). In addition, physical or emotional stressors activate the HPA axis to prevent substantial disruption of the organism's homeostatic balance (10). After activation of the HPA axis the hypothalamus releases CRH into the hypophysial-portal circulation which stimulates the biosynthesis and secretion of ACTH by the pituitary (9).

Within minutes ACTH increases the free cholesterol available to the adrenals, by stimulating steroidogenic acute regulatory protein (StAR) gene transcription (11, 12). The process of steroidogenesis starts in the mitochondria (13). In steroidogenic cells (cells that express P450scc) StAR moves cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (IMM) (13). On the IMM cholesterol is then available for the first rate-limiting step of steroidogenesis, mediated by P450scc (14). Steroidogenic factor 1 (SF-1) regulates transcription of StAR and several steroidogenic enzymes, including the rate limiting enzyme P450scc (15-17). SF-1 is mainly expressed in the adrenals, testes, ovaries, the hypothalamus, and the skin (17-21). Hypothalamic regulation is also dependent on SF-1. (20)

The final product of the HPA axis, glucocorticoids, present a negative feedback, causing repression of the release of CRH and ACTH and stopping steroid hormone synthesis (22). However, during stress this negative feedback loop is suppressed, leading to further increased ACTH secretion (9). In case of long-term stress, lasting days or weeks, high levels of ACTH lead to increased expression of steroidogenic enzymes and subsequently to adrenal cellular hypertrophy and hyperplasia (14).

1.1.3 Steroidogenic enzymes

Two classes of enzymes are involved in steroidogenesis: cytochrome P450 heme-containing proteins (CYP) and hydroxysteroid dehydrogenases (HSD) (Figure 1) (23, 24). While P450 mediated reactions are irreversible, HSD reactions could technically run in either direction, but in vivo each HSD enzyme opts for either predominantly performing oxidation or reduction reactions (23, 24).

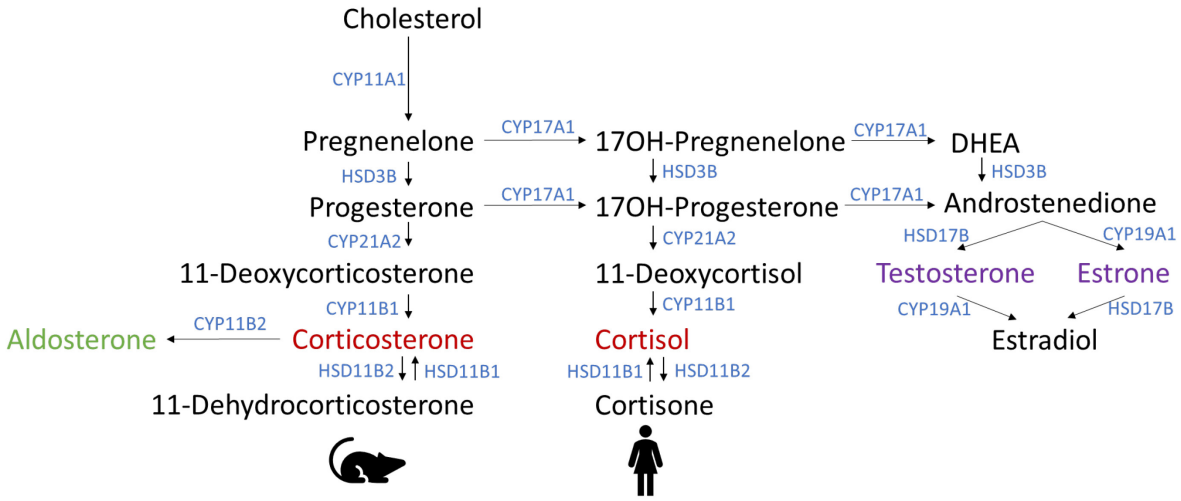


Figure 1 ***Steroid Synthesis Pathway.*** *An overview of the genes involved in the synthesis of glucocorticoids (red), mineralocorticoids (green), and sex hormones (purple). Since rodents do not express CYP17A1 their prevalent GC is corticosterone. In humans it is cortisol. Adapted from (25).*

CYP11A1 (P450scc) is the rate limiting enzyme of steroidogenesis. It catalyzes the first step in the synthesis of all steroid hormones, the conversion of cholesterol to pregnenolone. CYP11A1 is mainly expressed in all three zones of the adrenal cortex (zona fasciculata, zona reticularis and zona glomerulosa), ovary and testis (14, 26-29).

HSD3B (3βHSD) catalyzes a reaction, which converts pregnenolone to progesterone, 17α-hydroxypregnenolone to 17α-hydroxyprogesterone and dehydroepiandrosterone (DHEA) to androstenedione. HSD3B has multiple highly homologous isoforms. Humans have two isoforms, which are both involved in steroidogenesis. Human HSD3B2 is mainly expressed in the adrenal cortex and gonads. HSD3B1 is rather expressed in peripheral tissues, like skin and breast tissue. Mice have six isoforms, only 1 – 3 and 6 play a part in steroid hormone biosynthesis. Mouse HSD3B1 is equivalent to human HSD3B2 and is mostly expressed in the adrenal cortex and gonads, while the other isoforms are involved in steroidogenesis in peripheral sites (14, 26-30).

CYP17 (P450c17) catalyzes the conversion of pregnenolone and progesterone. Resulting in 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, intermediates in the biosynthesis of cortisol, or DHEA and androstenedione, precursors for gonadal hormones. CYP17 is expressed in the gonads and the human adrenal gland. In the human adrenal gland, it is expressed in the zona reticularis, site of androgen synthesis, and the zona fasciculata, site of glucocorticoid synthesis. However, it is not expressed in the zona glomerulosa, site of mineralocorticoid synthesis, since 17α-hydroxylation is not required for aldosterone synthesis. Neither is it expressed in mouse adrenal glands. The predominant glucocorticoid in mice is corticosterone rather than cortisol, which is favored in humans and the corticosterone synthesis pathway is closer to that of aldosterone (Figure 1) (14, 26-29).

CYP21 (21-hydroxylase, CYP21B in humans, cyp21a in mice) catalyzes reactions converting progesterone and 17α-hydroxyprogesterone to 11-deoxycorticosterone and 11-deoxycortisol, respective precursors for corticosterone and cortisol. This is a determining step in the biosynthesis of adrenal steroid hormones. CYP17 cannot react with 11-deoxycorticosterone, which therefore cannot be used in the biosynthesis of cortisol. The main site of expression for CYP21 is the adrenal cortex, where it plays an essential role in the biosynthesis of corticosterone, cortisol, and aldosterone (14, 26-29).

CYP11B1 (11β-hydroxylase) catalyzes a reaction that yields active glucocorticoids, corticosterone from 11-deoxycorticosterone and cortisol from 11-dexocortisol. *CYP11B2* or aldosterone synthase is responsible for aldosterone synthesis from 11-deoxycorticosterone. CYP11B1 and B2 are mainly expressed in the adrenal cortex, CYP11B2 in the zona glomerulosa and CYP11B1 in the zona fasciculata and zona reticularis. Humans have a higher expression of CYP11B1 whereas in mice both enzymes show similar levels of expression (14, 26-29).

CYP19 (aromatase) catalyzes the last step in the biosynthesis of estrogens and is mainly expressed in the gonads (14, 26-29).

HSD17B (17βHSD) is relevant for the final step of the synthesis and activation of estradiol and testosterone. In humans there are 9 different forms of HSD17B, three of which are involved in the synthesis of estradiol and testosterone, namely types 1, 3, and 7. HSD17B is mainly expressed in the gonads and mammary gland (14, 26-29).

HSD11B (11βHSD) does not contribute to de novo synthesis of steroids. It has two isoforms, HSD11B1 activates and HS11B2 inactivates glucocorticoids. In peripheral tissues like the lung, HSD11B1 allows reactivation of inactive metabolites of cortisol or corticosterone (14, 26-29).

1.2 Intestinal Steroidogenesis

Intestinal steroidogenesis acts independently of the hormones of the HPA-axis (31).
The intestine produces glucocorticoids via de novo synthesis from cholesterol, as opposed to sites of extra-adrenal glucocorticoid production like the lung, which reactivates inactive metabolites circulating in the serum (1, 32). In the intestine the same enzymes as in the adrenals are involved steroid synthesis, but their transcriptional regulation is different (33). Expression of steroidogenic enzymes and cortisol production have been shown in human colonic epithelial cells (35, 36). Steroidogenesis in the intestine takes place in intestinal epithelial cells, more specifically in proliferating cells at the base of the crypts (37). As the cells mature and ascend to the surface, their steroidogenic capacity fades (Figure 2) (32).

SF-1, a nuclear receptor responsible for transcriptional regulation of glucocorticoid synthesis in the adrenal glands, is not expressed in the intestine (34, 35). Instead liver receptor homolog-1 (LRH-1), a close homolog to SF-1, takes its place and is involved in the transcriptional regulation of intestinal steroidogenesis (35, 36). LRH-1 shows similar patterns of expression to steroidogenic enzymes (31).

The contribution of extra-adrenal GC synthesis to systemic levels appears to be minimal compared to that of the adrenal gland, considering an almost undetectable GC concentration in the serum following adrenalectomy (37). Analysis of ex-vivo murine organ cultures showed a base corticosterone production of 17 ng per hour for the adrenals. Immunological stress could increase this rate up to 100 ng per hour. In comparison, the small intestine produced 2,5 ng corticosterone per hour (1). Human colonic tissue seems to have a similar capacity for cortisol production (38).

Extra-adrenal GC synthesis is important for regulating local inflammatory processes and maintaining homeostasis (39). Extra-adrenal GCs play a role in the differentiation of intestinal epithelial cells, as well as maintaining the intestinal epithelial barrier and tight junctions (40, 41). Baseline levels of the rate limiting steroidogenic enzyme P450scc are rather low, but can be induced by immunological stress (37).

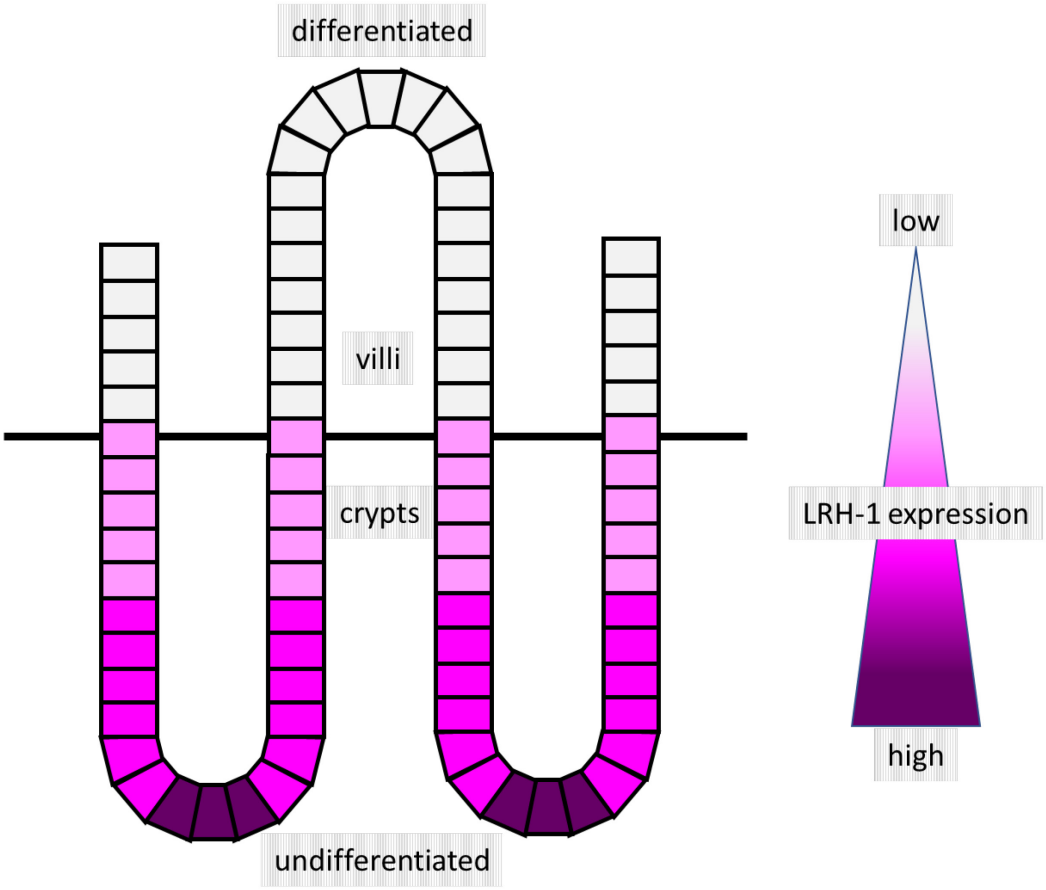


Figure 2 ***LRH-1 expression in the intestinal epithelium.*** *At the bottom of the crypt cells are undifferentiated whereas towards the surface cells are more differentiated. LRH-1 expression correlates with markers of undifferentiated cells and shows decreased levels with increasing differentiation. Therefore, LRH-1 expression is highest at the bottom among undifferentiated cells (42). Adapted from (42).*

1.3 Nuclear Receptors

Nuclear receptors (NRs) are central regulators of steroidogenic enzymes. NRs are a special class of transcription factors, which are responsible for the activation or repression of specific genes by modulating their transcription (43). NRs bind to promoters of their respective target genes. Ligand binding to NRs recruits transcriptional co-activators or releases transcriptional co-repressors, which leads to conformational changes in the DNA at the transcription start sites and results in gene transcription (5).
The 48 NRs known in humans are categorized in 7 subfamilies (NR0-NR6) (44, 45). Multiple physiological processes are regulated by NRs including inflammation, metabolism, reproduction and the circadian rhythm (46, 47). Because dysregulation of NRs contributes to various diseases like obesity, cancer and diabetes, certain NRs have become popular pharmaceutical targets (48, 49).

The modular domain structure is fairly uniform among NRs. NRs contain five domains, A-E (Figure 3) (50, 51).

- A/B, the N-terminal domain (NTD), contains the activation function-1 region (AF-1), which interacts with co-regulators (52). The NTD is a target for modifications like acetylation or phosphorylation, that drive or inhibit transcription (53).
- C, the DNA binding domain (DBD), consists of two subdomains (54). One subdomain is responsible for DNA reading and base-specific interactions with the DNA (55). The other one connects to the phosphate backbone of the DNA (5).
- D is a flexible hinge between the DBD and the ligand binding domain (LBD) (56).
- E, the ligand binding domain, binds to ligands and interacts with co-regulators (57, 58). The LBD contains a ligand binding pocket, which varies among NRs and allows recognition of a wide array of different ligands between NRs (50, 59). The LBD contains the activation function-2 region (AF-2), which facilitates interaction with different co-regulators upon ligand binding (57, 59).

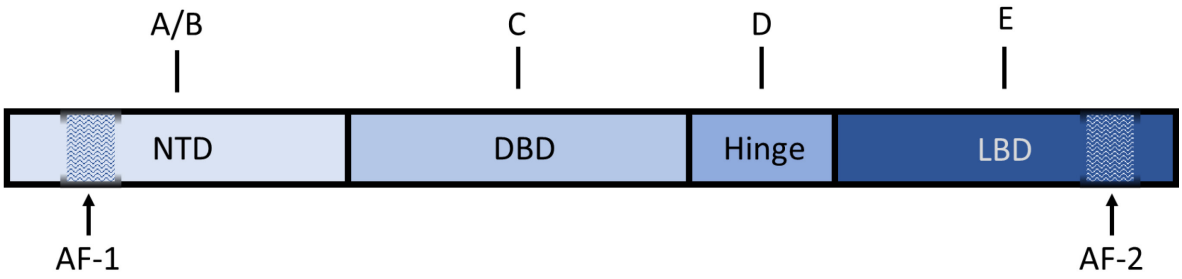


Figure 3 ***Basic structure of a nuclear receptor.*** *Adapted from (5).*

For 24 of the 48 NRs ligands have been identified. NRs without a known ligand are classified as orphan receptors or adopted orphan receptors if a likely ligand has been discovered (5). Ligand binding stabilizes the LBD and promotes the binding of co-regulators (60).
When binding with DNA most NRs form homodimers or heterodimers with the retinoid-X-receptor (RXR) (61). Only few NRs, like the members of the NR5A subfamily, function as monomers due to high-affinity binding to their target genes (62, 63). After DNA binding, co-regulators come into effect, acting either as activators or inhibitors at the AF-1 and AF-2 sites, respectively (52, 61, 64).

Based on their mechanism of action, NRs can be divided into 4 subtypes (Figure 4):

- Type 1 NRs (e.g. GR) remain in the cytoplasm bound to a chaperone protein and are activated by steroidal hormones like glucocorticoids (65). When activated by a ligand they leave their chaperone protein and move to the nucleus, where they bind as homodimers to the DNA (66, 67).
- Type 2 NRs (e.g. FXR) stay in the nucleus regardless of their activation state (68). Upon activation by a ligand, a co-repressor is swapped with a co-activator and the receptor binds to the DNA as a heterodimer with RXR (69).
- Type 3 NRs (e.g. RXR) work like Type 2 NRs, but instead form homodimers (54).
- Type 4 NRs (e.g. LRH-1, SF-1) work like Type 2 NRs, but instead bind as monomers to the DNA (70, 71).

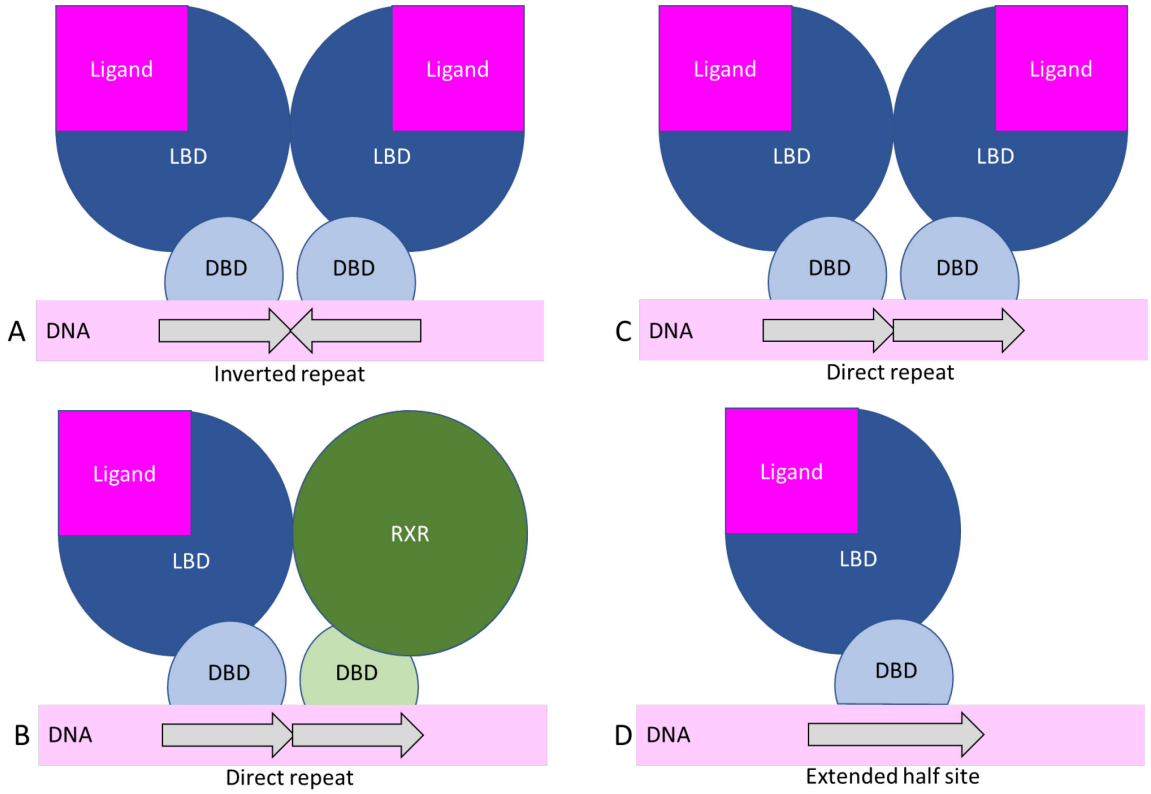


Figure 4 ***Mechanism of action of different NRs.*** *A: Type 1 NRs bind as homodimers to DNA response elements consisting of two inverted repeats (palindromes). B: Type 2 NRs bind as heterodimers with RXR to direct repeat DNA response elements. C: Type 3 NRs bind as homodimers to direct repeat DNA response elements. D: Type 4 NRs bind as monomers to extended half sites on the DNA response elements. LBD: Ligand binding domain, DBD: DNA binding domain, RXR: retinoid-X-receptor. Adapted from (5).*

Family	Nuclear Receptor	Ligand
0B	DAX1	Orphan
0B	SHP	Orphan
1A	TR α, β	Thyroid hormones
1B	RAR α, β, γ	Retinoic acids
1C	PPAR α, β, γ	Fatty acids
1D	REV-ERB α, β	Heme
1F	ROR α, β, γ	Sterols
1H	FXR α, β	Bile acids
1H	LXR α, β	Oxysterols
1I	VDR	Vitamin D
1I	PXR	Endobiotics and xenobioticcs
1I	CAR	Xenobiotics
2A	HNF4 α, γ	Fatty acids
2B	RXR α, β, γ	Retinoic acid
2C	TR 2,4	Orphan
2E	TLX	Orphan
2E	PNR	Orphan
2F	COUP-TF α, β, γ	Orphan
3A	ER α, β	Estrogens
3B	ERR α, β	Orphan
3C	AR	Androgens
3C	GR	Glucocorticoids
3C	MR	Mineralocorticois and glucocorticoids
3C	PR	Progesterone
4A	NGF-1B	Orphan
4A	NURR1	Unsaturated fatty acids
4A	NOR-1	Orphan
5A	SF-1	Phospholipids
5A	LRH-1	Phospholipids
6A	GCNF	Orphan

Table 1 ***Overview of nuclear receptors and their respective ligands.*** *Adapted from (5).*

1.4 LRH-1 (NR5A2)

LRH-1 (NR5A2) belongs to the class of orphan receptors, but phospholipids as ligands have been proposed (see next chapter). LRH-1, just as SF-1, does not necessarily need a ligand for a baseline level of activity, because it is constitutively active (17). While LRH-1 displays the basic structure of a nuclear receptor, there are a few distinct LRH-1-specific features (17). First, the N-terminal domain of LRH-1 does not contain the activation function-1 region (17, 72). Second, LRH-1 contains an FTZ-F1 box, which is an extension of the DBD. This domain assists the high-affinity binding to the DNA of their target genes and facilitates a stable connection as a monomer (17, 62).

Binding to a ligand leads to conformational changes of the LBD to allow activation of the NR. If the ligand-binding pocket of a NR is empty, the LBD openly presents a docking surface for a co-repressor. After binding to an agonist ligand, this surface is blocked from binding to co-repressors. Instead a new surface intended for co-activators is formed (17, 73). LRH-1 has a conformation as if it is bound to an agonist ligand, even in the absence of a ligand (17, 74).

Small heterodimer partner (SHP) and farnesoid-X-receptor (FXR) are examples of co-repressors of LRH-1 (17, 75, 76). Co-activators of LRH-1 include multiprotein bridging factor (MBF1) and β-catenin (17, 77, 78).

Various tissues show expression of LRH-1. It is expressed primarily in the gastrointestinal tract such as the intestine, pancreas and liver but also in reproductive organs such as testis, ovary, placenta, and endometrium (17, 72, 79). Other sites of LRH-1 expression include the adrenal gland, pituitary and hypothalamus (17, 80, 81).

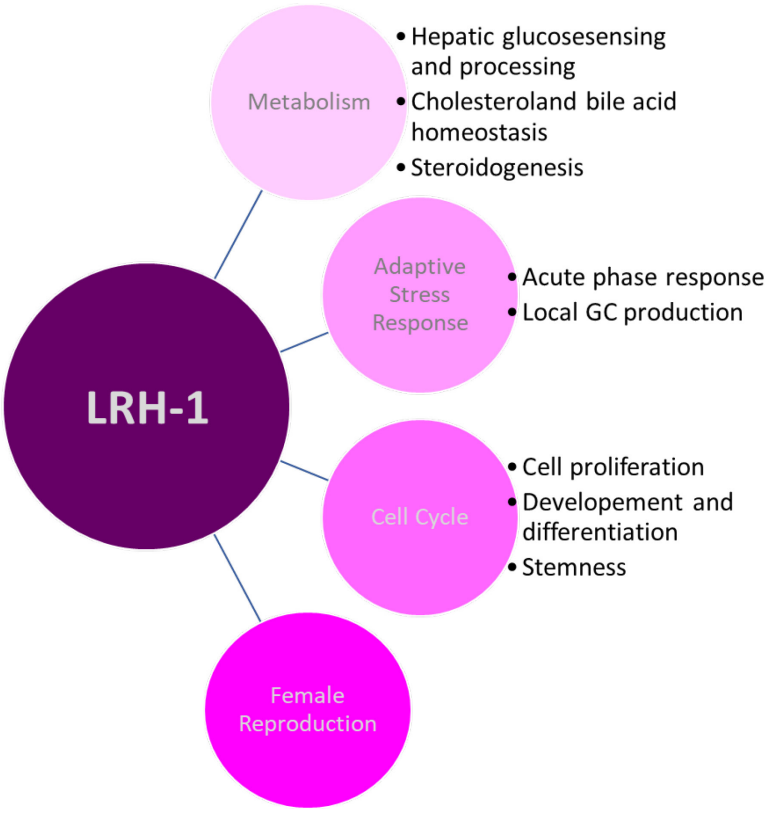


Figure 5 ***Overview of the general functions of LRH-1.*** *Adapted from (82).*

1.4.1 Potential Ligands for LRH-1

LRH-1 does not need a ligand for basal activity, as its LBD is stable even without a ligand (17, 74, 83). However, phospholipids have been identified as ligands for LRH-1 in in vitro and in vivo models (84). Phospholipid binding to the LRH-1 LBD does not cause conformational changes within LRH-1 but does affect transcription (17, 85). Phospholipids, such as phosphatidylcholine, have not only been shown to increase the transcriptional activity of LRH-1 but in addition phospholipid binding stabilizes the NR and reduces co-repressor binding (17, 74, 86).

Pharmacological ligands for LRH-1 exist, e.g. the pharmacological ligand dilauryol-phosphatidyl-choline (DLPC) activates LRH-1 and improves glucose levels in diabetic mice (17, 84). Endogenous ligands have yet to be discovered, however, it is disputed whether phospholipids could be endogenous ligands (17, 87). Interestingly, inhibition of one of the two cellular pathways generating endogenous phospholipids, the PEMT pathway, which produces a distinct class of phospholipids, reduces LRH-1 signaling (88).

In contrast, inhibition of the other pathway, the Kennedy pathway, which endogenously generates the bulk of phospholipids, does not affect LRH-1 signaling (88).
Two inverse agonists of LRH-1 have been identified to block the constitutive activity of LRH-1. These inverse agonists, ML179 and ML180, remove LRH-1 from the nucleus and shuttle it into the cytoplasm preventing it to perform transcription (17, 89).

1.4.2 LRH-1 and Steroidogenesis

LRH-1 is critically involved in intestinal glucocorticoid (GC) synthesis and immune homeostasis (36). LRH-1 regulates transcription of steroidogenic genes, including CYP11A1, CYP11B1, HSD3B, CYP17A1 and StAR (15, 17).
T-cell activation during immune responses leads to increased intestinal expression of LRH-1, CYP11A1 and CYP11B1 and subsequently to increased glucocorticoids synthesis in the intestine (33). In LRH-1 haplo-insufficient mice, which express only one functional LRH-1 allele, no such increase can be observed (33). This demonstrates that LRH-1 is required for the regulation of intestinal steroidogenesis, at least under inflammatory stress conditions. Consequently, intestinal LRH-1 deficient mice with reduced intestinal GC content are more prone to injury by experimentally induced colitis (36).

1.4.3 LRH-1 and Cell Cycle

Apart from its steroidogenic involvement, LRH-1 also plays a role in the cell cycle of intestinal epithelial cells. It regulates the expression of various cell cycle-regulating genes. LRH-1 drives cell cycle progression and promotes the renewal of intestinal cells by the activation of cyclin E1 as well as induction of cyclin D1 and c-Myc transcription, all facilitated by the interaction of LRH-1 with β-catenin (90).
Heterozygous LRH-1 +/- mice, with reduced mRNA levels of LRH-1 throughout the entire intestine, show reduced crypt length with otherwise normal intestine morphology (90). Similar to LRH-1, mRNA levels of c-Myc and cyclin D1 as well as several markers of cell renewal are reduced in the intestine of LRH-1 +/- mice (90). Of note, homozygous LRH-1 -/- mice die in utero, due to the fundamental role of LRH-1in developmental stages (90).

1.4.4 LRH-1 and Tumorigenesis

The possible involvement of LRH-1 in tumorigenesis is double-edged. On the one hand LRH-1 modulates inflammation, with chronic inflammation being a risk factor for intestinal cancer (91, 92). On the other hand, LRH-1 cooperates with β-catenin in promoting cell renewal (90, 92). β-catenin also plays a central role in the formation of colon cancer. The adenomatous polyposis coli (APC) gene usually facilitates the degradation of β-catenin. In case of a mutated APC gene β-catenin is no longer degraded and accumulates. This results in out of control cell proliferation and leads to potentially cancerous intestinal polyps.
APC gene mutations are the cause of the hereditary disease familial adenomatous polyposis (FAP). Patients with FAP suffer from numerous colon polyps starting from a young age and have an increased risk for colon cancer (92, 93).

A study using two different intestinal cancer models in mice, a heterozygous APC Mutation (APCMin/+) and application of the carcinogen azoxymethane (AOM), gave insight into the role of LRH-1 in tumorigenesis (92). Haploinsufficient LRH-1 +/- mice were compared to LRH-1 +/+ mice with normal LRH-1 expression. In both models LRH-1 +/- mice showed a significant decrease of tumor numbers in the intestine. In the AOM challenged group cell proliferation and DNA synthesis was lower in LRH-1 +/- mice compared to LRH-1 +/+ mice. Since AOM is a model for the early stages of tumorigenesis, this data suggests that LRH-1 plays a critical role in the early phases of tumor formation (92).
In both models LRH-1 mRNA levels were surprisingly decreased in tumors compared to healthy intestinal samples. Conversely, mRNA levels of TNF-α, a proinflammatory cytokine, were increased in tumors. This occurred in both LRH-1 +/+ and LRH-1 +/- mice, although LRH-1 +/- mice showed an overall decrease of TNF-α levels compared to LRH-1 +/+ mice (92). Overall, protection against tumor formation in LRH-1 haploinsufficient mice might stem from decreased cell proliferation as well as modulation of the inflammatory response. Furthermore, colon cancer cells have been reported to produce active GCs in an LRH-1 dependent manner. This might present a tumor escape mechanism in colon cancer, as these GCs suppress T-cell activation (94).

In healthy human intestinal epithelial cells LRH-1 protein is mainly expressed in the nucleus of cells in the basal part of the crypts with no expression in cells at the surface of the intestine (92). Thus, LRH-1 expression is contained to undifferentiated proliferating cells. In healthy intestinal tissue surface epithelial cells are mature non-proliferative cells.
Human intestinal tumor samples show increased LRH-1 protein expression in the cytoplasm of crypt cells and abnormal expression occurring in the cells at the surface (92).

Not only the cell cycle genes, cyclin E1, cyclin D1 and c-Myc, which are all in part regulated by LRH-1 and β-catenin, tend to be overexpressed in gastrointestinal cancerous tumors in humans, but also LRH-1 itself is overexpressed in different cancers (90, 95).
LRH-1 is also implicated in extra-intestinal cancers. Pancreatic cancer cell lines show higher mRNA levels of LRH-1 compared to physiological pancreatic cells (96). Overexpression of LRH-1 in pancreatic cancer cells in mice has been found to be associated with increased cancer growth and metastasis (97). In breast cancer cells overexpression of LRH-1 promotes cancer cell growth, invasion, and estrogen receptor expression (95). Ovarian granulosa cell tumors show increased expression of LRH-1 compared to healthy ovarian tissue (17).

1.4.5 LRH-1 and Inflammatory Bowel Disease

LRH-1 affects severity of inflammatory bowel disease (IBD) in experimental models in mice. There is also an indication of its involvement in human IBD. In a study by Coste et al., with LRH-1 +/- haploinsufficient mice and LRH-1 +/+ as control, 2,4,6-trinitrobenzene sulfonic acid (TNBS) and dextran sodium sulfate (DSS) were administered to simulate human IBD (36). TNBS, applied intrarectally to induce acute colitis, triggered more severe inflammation in the colon of LRH-1 +/- mice (36). DSS treatment, inducing less acute colitis, revealed a reduced capacity for regeneration of the intestinal epithelium on top of more severe inflammation in the colon of LRH-1 +/- mice (36). LRH-1 seems to have protective properties in a state of inflammation and mediate cell regeneration (36). The steroidogenic genes CYP11A1 and CYP11B1 were reduced on the mRNA level and corticosterone release was decreased in TNBS treated LRH-1 +/- mice (36). Glucocorticoids like corticosterone exert immunosuppression by promoting expression of anti-inflammatory genes and inhibiting expression of proinflammatory proteins (36, 98). Mice, that tissue-specifically do not express LRH-1 in the intestine, but have normal extra-intestinal LRH-1 expression, also

show reduced CYP11A1 and CYP11B1 mRNA levels and reduced corticosterone levels in the colon after TNBS treatment (36).

Human IBD is also associated with reduced LRH-1 levels. Patients with Crohn's Disease (CD) or Ulcerative Colitis (UC) showed decreased LRH-1 expression in inflamed parts of their colon, compared to healthy mucosa (37). This decrease was not caused by necrosis or loss of epithelial cells (36, 99). CYP11A1 and CYP11B1 mRNA levels were also decreased in inflamed parts of the colon of IBD patients, in comparison to healthy parts of the same colon or healthy control patients (36). The decrease of peroxisome proliferator-activated receptor γ (PPARγ), an anti-inflammatory factor, correlated with impaired intestinal steroidogenesis in UC patients (100). These findings could indicate the involvement of steroidogenic regulation in the pathogenesis of human IBD (36).

One study reviews the application of phosphatidylcholine (PC), a certain phospholipid species. PC was found to be reduced in the intestine of UC patients. The local administration in an experimental treatment with PC was found superior to placebo in steroid-refractory UC patients due to its anti-inflammatory properties (101).

A defect of the tight junction barrier (TJB) in the intestine might contribute to the symptoms of CD (41). Treatment of CD with GCs can improve intestinal TJB defects, which correlates with clinical improvement (41, 102). A promising treatment option for active inflammation in CD patients is normalizing permeability of the intestinal TJB (41). Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, is elevated in the inflamed intestine of CD patients (41, 103). TNF-α is known to increase TJB permeability (41, 104). Boivin et al analyzed the effect of GCs on TNF-α induced leaky TJB in Caco-2 cells. TNF-α increases permeability in Caco-2 cells by increasing myosin light chain kinase (MLCK) expression (41, 105). GC application did not affect baseline TJB permeability, but it prevented an increase of permeability after TNF-α administration (41). The GC maintained the TJB by activating the GR which in turn prevented TNF-α from promoting MLCK expression (41).

1.5 The Gut Microbiome

The gut microbiome consists mostly of bacteria. About 1000 species of intestinal bacteria are known (106). Composition of the microbiome varies greatly between individuals. Each person has approximately 150 of these 1000 known species in their intestine (106). There are some differences between small and large intestine. For one, bacterial load in the colon is up to 1 billion times higher compared to parts in the duodenum with the lowest bacterial load, and the thickness of the mucus layer, an integral component to maintain homeostasis, increases towards the colon (107, 108).

Humans and their microbiome usually live together in symbiosis. The commensal bacteria developed strategies to avoid being targeted by the host's immune system and maintain homeostasis (109). The gut microbiome helps with nutrient breakdown and absorption as well as protection against pathogens. They produce metabolites, such as butyrate, which help maintain the epithelial barrier and modulate inflammation and the immune response (109). The microbiome is also involved in estrogen metabolism by activating conjugated estrogens excreted in bile (109). In addition, the microbiome affects regulation of steroidogenic enzymes in the adrenals and the intestine. Vagnerová et al. showed upregulation of CYP11A1 and HSD3B1 in the adrenals and upregulation of LRH-1 and HSD3B2 in the intestine of mice with intact microbiome compared to germfree mice (110). However, genes responsible for the regulation of the HPA-axis do not seem to be affected by microbiota (110).

An imbalanced, dysbiotic gut microbiome however can disturb the homeostasis between bacteria and immunoregulatory mechanisms and is associated with a range of diseases, not limited to the intestine (108). Therefore, strategies to rebalance the gut microbiome and consequently modulate the course of diseases like IBD are a popular field of investigation.

Fecal microbiota transplantation (FMT) is an established treatment for recurring clostridium difficile infections (111, 112). UC patients have a less diverse gut microbiome than healthy controls (111, 113). So far experimental FMT in UC patients has shown promising results in inducing remission and might prospectively provide an additional treatment option for UC (111). Although the immunological effects of FMT are not yet fully understood LRH-1 interaction and steroidogenesis might play a role in these effects. Moreover, the metabolome, which is produced by the local microbiome might generate an agonistic or antagonistic ligand for LRH-1 und thus directly regulate steroidogenic transcription.

2 Hypothesis and Aims

Glucocorticoids are not only produced in the adrenal glands but also locally generated in extra-adrenal tissues including the intestine, where they mediate localized physiological effects. Intestinal steroidogenesis is regulated on transcriptional levels by the nuclear receptor LRH-1. The natural ligand and in particular the source of the natural ligand for intestinal LRH-1 are unknown. Identifying the gut microbiome as source of a natural LRH-1 ligand and consequently as a modulator of local intestinal steroidogenesis would be a novel link in understanding signaling pathways of the gut microbiome and may have implications in treatment concepts for inflammatory intestinal processes.
We therefore overall hypothesize that changes in the gut microbiome affect local intestinal steroidogenesis and that these effects are mediated via LRH-1 signaling.

Aim: Test LRH-1 target genes and steroidogenic genes in the intestine of germfree and conventional mice.

3 Material and Methods

3.1 Primer design

For primer design I used http://primer3.ut.ee. As a source I used the respective genome sequences I received from https://genome.ucsc.edu. Upon primer selection I focused on primers that were located on different exons on the coding sequence, had a size of approximately 20 bases, similar melting temperatures close to 59°C, a product size in the range of 50-200 bp and low self or pair complementarity. I checked all primers in UCSC's In-Silico PCR.

3.2 Primers (mouse)

Gene	**Forward**	**Reverse**
Tbp	ctctggaattgtaccgcagc	agttgtccgtggctctctta
Lrh-1 (114)	gtgtggcgataaagtgtctg	ttggcaattctggttctctatg
Cyp11a1 (36)	gtcggaaggtgtagctcagg	cactggtgtggaacatctgg
Cyp11b1	ctgggacgtggtgtgttctt	cccttgctatcccatccacc
Hsd3b1	gggcatctctgttgtcatcc	cttgaacacaggcctccaat
Hsd3b3	ggacaagcatcaaggtgacag	ccctgcaacgtcaactgag
Shp	aagggcacgatcctcttcaa	gtaccagggctccaagact
Fgf15	gaggaccaaaacgaacgaaatt	acgtccttgatggcaatcg
Gck (115)	acattgtgcgccgtgcctgtgaa	agcctgcgcacactggcgtgaa
ApoA4	ttcctgaaggctgcggtgct	cagctgtacgacaaagggca

Table 2 ***Mouse Primers***

3.3 Antibodies

Antibody	Product #	Company	Host
3ß-HSD (A-1)	sc-515120	Santacruz Biotechnology, Inc	mouse
CYP11A1 Polyclonal AB	PA5-37359	ThermoFisher Scientific	rabbit
CYP11B1	NBP1-68883	Novus Biologicals	rabbit

Table 3 ***Antibodies***

3.4 Mouse Samples

We received samples of jejunum, ileum, and colon from conventional and germfree (n=5 each) mice from our collaborator, Prof. Hanns-Ulrich Marshall, Institute of Medicine, University of Gothenburg, Sweden (116).

3.5 RNA-isolation

A third of each jejunum, ileum and colon was put in trizol and homogenized in the MagnaLyser using MagNALyser Green Beads. Chloroform was added to the samples and after 15 minutes on a chilled rotary disc they were centrifuged for 15 minutes (13000 rpm, 4°C). The clear phase on top (containing RNA) was taken off and isopropanol was added to it. After two minutes on ice the samples were centrifuged again for 15 minutes (13000 rpm, 4°C). The liquid was taken off and discarded. 100% Ethanol was added to the pellet and the samples were centrifuged again for 15 minutes (13000 rpm, 4°C). The liquid was taken off and discarded and the pellets were washed with 70% ethanol twice. After that they were left to dry for 5 minutes. Aqua dest. was added to the pellets and they were put in the heater to solubilize the RNA. After 10 minutes at 55°C they were put on ice for at least 30 minutes. RNA concentration was measured using the NanoDrop2000 spectrophotometer.

3.6 cDNA

cDNA was synthesized with SuperScript II Reverse Transciptase, Random Hexamer, 0.1M DTT, 5X First Strand Buffer, RNAse inhibitor, dNTPs and Aqua dest. using the BIO-RAD MyCyler Thermal Cycler (Protocol: 42°C for 90‘, 70°C for 15‘, 4°C for ∞).

3.7 qPCR

For the mastermix Blue S’Green qPCR 2x Mix, Aqua dest. and respective primers were mixed. In each well of a 384 well plate 8µL mastermix and 2µL undiluted cDNA were distributed. qPCR was performed on a Roche Light cycler with the following protocol: 95°C for 10’, 40 cycles: 95°C for 10’’ and 60°C for 1’.

3.8 Protein Isolation

Samples were put in homogenizing buffer with proteinase-phosphatase inhibitor (1 tablet proteinase-phosphatase inhibitor in 10 ml homogenizing buffer). Homogenizing buffer was made by dissolving 42,8 g saccharose in a small volume of A. dest, adding 5ml HEPES-KOH (pH=7,5) and 1m 0,5M EDTA, and filling everything up with A. dest ad 500ml.
The tissue was treated with ultrasound 2x for a total of 10 seconds to disconnect and homogenize the tissue and put on ice in between. The mixture was then centrifuged for 1 minute at 3000 rpm, the supernatant was taken off and protein concentration was measured with a BCA-Kit Protein Assay Kit (Thermo Scientific) using a BSA standard curve and the color change was measured on SPECTROstar (Omega) photometer.

3.9 Western Blot

Gel preparation

10% stacking gel solution (3,69 ml A. dest, 2,97 ml 30% Acrylamid, 2,25 ml stocking gel stock, 90 µl 10% SDS, 24,3 µl APS, 8,1 µl Temed) was prepared and filled in between glass plates with a thickness of 1,5 mm. APS and Temed were added right before use. A. dest was added on top and the gel was left to solidify for 40 minutes. After the stacking gel was

polymerized, the A. dest on top was discarded and excess water was gently removed with filtering paper. 4,5% separating gel solution (2,95 ml A.dest, 0,75 ml 30% Acrylamid, 1,25 ml stacking gel stock, 50 µl 10% SDS, 20 µl APS, 6,5 µl Temed) was applied on top of the stacking gel and a comb was inserted. The gel was left to solidify for 20 minutes. The gel was placed in an electrophoretic chamber, filled to the top with 1x running buffer for at least one hour or overnight.

- Stacking gel stock: 36,3 g Tris diluted in 50 ml A. dest, adjust pH to 8,8 with HCL, add 0,8 g SDS, fill with A.dest ad 200 ml and sterile-filter.
- Seperating gel stock: 12 g Tris diluted in 50 ml A. dest, adjust pH to 6,8 with HCL, add 0,8 g SDS, fill with A.dest ad 200 ml and sterile-filter.
- 10x running buffer: 60 g Tris, 288 g glycerin, 20 g SDS ad 2 liters with A. dest, pH=8,6.

Sample preparation

Sample buffer (340 µl 3x sample buffer + 60 µl β-Mercaptoethanol and diluted with A. dest for 1x sample buffer) was added to the samples. The samples were heated up to 95°C and then put on ice.

- 3x sample buffer: 3,75 ml separating gel stock, 6 ml 10% SDS, 7,32 ml 100% glycerol, a few crumbs of bromophenol blue.

Loading gel

On the first position the ladder was loaded. From the second position the samples were loaded, 10-45 µg protein in a volume of 5-10 µl per well. First the gel ran at 200 volts for five minutes until the samples were collected at the bottom of the well. Then it ran at 100 volts for about 1,5 hours until the loading dye had reached the bottom of the gel.

Transfer

The gel was released from the glass plate and put on nitrocellulose membrane between two filtering papers and a sponge on each side. Then it was put back in an electrophoretic transfer chamber, filled to the top with transfer buffer (2,8 g TRIS + 14,3 g glycin dissolved in 800 ml A. dest. + 200 ml methanol). Transfer happened at 100 volts for 1 – 1,5 hours, packed in ice.

Antibodies

After the transfer, the membrane was stained with Ponceau S for 1 minute to verify even protein loading and visualize the protein ladder. After rinsing with A. dest. the ladder was labeled to locate the correct size of the proteins. Then the membrane was put in 5% skimmed milk in TBS-T for at least one hour to block non-specific binding and reduce background. The first antibody, diluted in 5% skimmed milk in TBS-T, was applied and incubated overnight. The next day it was rinsed in TBS-T (3x5 minutes). The second antibody, diluted in TBS-T, was applied, and incubated for one hour at room temperature. Then it was rinsed in TBS-T (3x15 minutes). For imaging the blots Biorad clarity Western ECL substrate was applied to the blots to visualize bound antibodies. The signal was captured in a ChemiDoc analysis apparatus (Biorad).

3.10 Immunofluorescence

Flash-frozen samples were cut into 5 µm cryosections in a cryo-microtome and sections were put on glass slides. Then they were fixed according to the respective antibody protocol (CYP11A1 in 4% formol, methanol and acetone / CYP11B1 in acetone). The first antibody (1:50) was applied for 1 hour. Then the samples were rinsed in PBS (3x3 minutes). The second antibody was applied in a dark room for 30 minutes. Again they were rinsed in PBS ensuing a short rinse in A.dest. Then they were cover-slipped with fluorescent mounting medium containing DAPI (prolong gold antifade reagent with DAPI, Invitrogen). Imaging was performed on a Confocal Laser Scan Microscopes (LSM510 Meta, Nikon A1R).

3.11 Statistics

Data are expressed as mean values and standard deviation of mean. All experiments were performed at least twice with a minimum of two replicates. Statistical analysis was carried out in Excel and SigmaPlot 11.0. Statistical significance was determined using t-test. A p-value of <0.05 was considered statistically significant.

4 Results

4.1 Expression of Steroidogenic Enzymes along the Intestinal Tract

Several studies have determined expression of steroidogenic enzymes in the intestine. However, to the best of our knowledge there are no studies yet, which determine expression levels in different parts of the intestine. Thus, the relative difference of expression levels between the small and the large intestine has not yet been determined. This is relevant since also the content and composition of the gut microbiome significantly differs between different parts along the gastrointestinal tract. All qPCR results were normalized to household gene TATA-box binding protein (TBP).

4.1.1 LRH-1

We first determined the expression profile of the transcriptional regulator LRH-1.
LRH-1 is expressed along the entire intestine (Figure 6). Highest levels of expression were shown in ileum and colon. Expression in jejunum was about 40% less than the expression in colon. There was no significant difference between germfree and conventional mice.

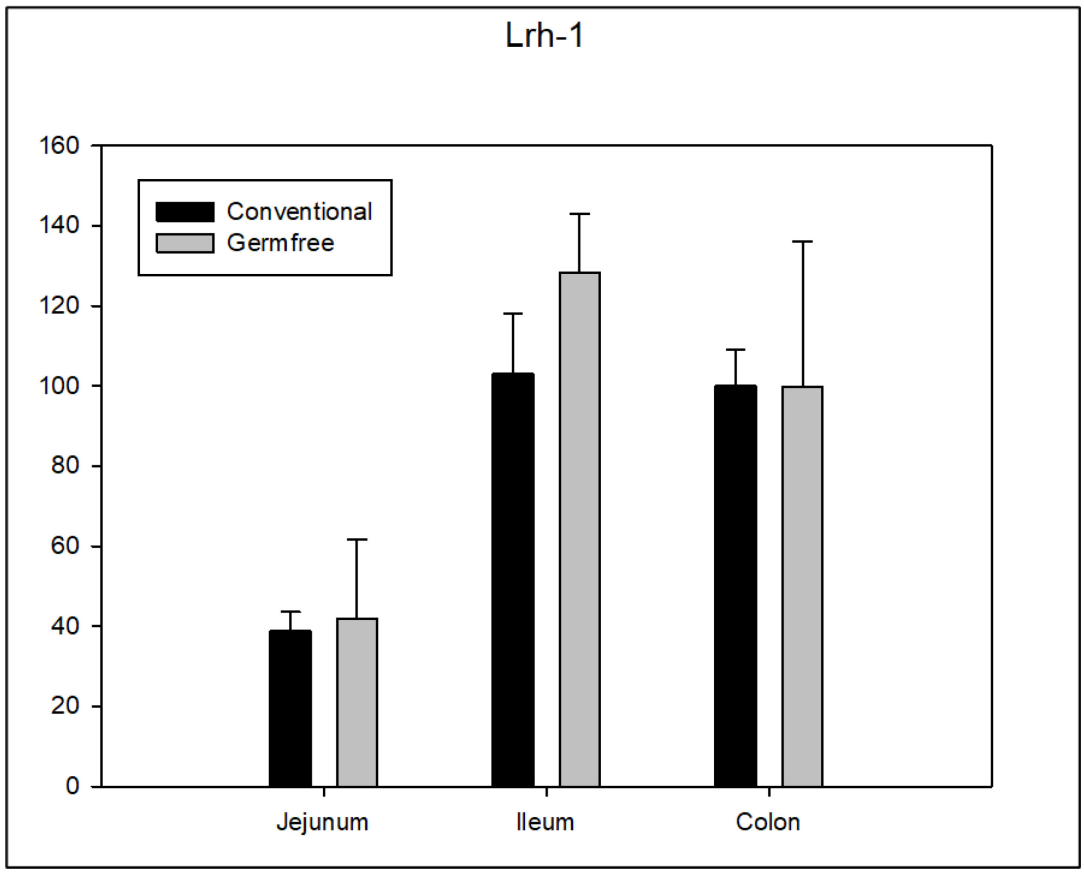


Figure 6 ***qPCR of LRH-1 in the intestine of conventional and germfree mice Results are displayed in % of conventional colon. n=5 mice for each condition.***

4.1.2 CYP11A1, CYP11B1, HSD3B1 and HSD3B3

The mRNA levels of steroidogenic target gene CYP11A1 were at the detection limit at Cp 30. CYP11B1 was too lowly expressed and was not detectable in qPCR at all. Even after re-performing analysis using published primer sequences and using higher amounts of cDNA there was no reliable signaling detectable.

In mice there are six known isoforms of HSD3B, in humans there are two. We analyzed expression of HSD3B1 and HSD3B3 along the mouse intestine. Mouse HSD3B1 is equivalent to human HSD3B2 and is mainly expressed in classical steroidogenic tissues such as the adrenals and gonads. Out of the other five murine isoforms HSD3B3 is critically involved in extra-adrenal steroidogenesis and seems to be the closest analog to human HSD3B1.

In our analysis HSD3B3 was the dominating isoform, with higher expression levels than HSD3B1 along the entire intestine. The highest expression of both, HSD3B1 and HSD3B3, along the intestine was observed in the colon, where expression levels were approximately 10- to 30-times higher compared to the small intestine (Figure 7).

The microbiome status, i.e. germfree or conventional mice, had a significant impact on gene expression levels. HSD3B1 and HSD3B3 showed a significant two- to four-fold increase in the small intestine of germfree mice compared to conventional mice. However, in the colon, where expression levels were highest the lack of gut microbiome in germfree mice significantly reduced HSD3B1 and HSD3B3 expression levels (Figure 7).

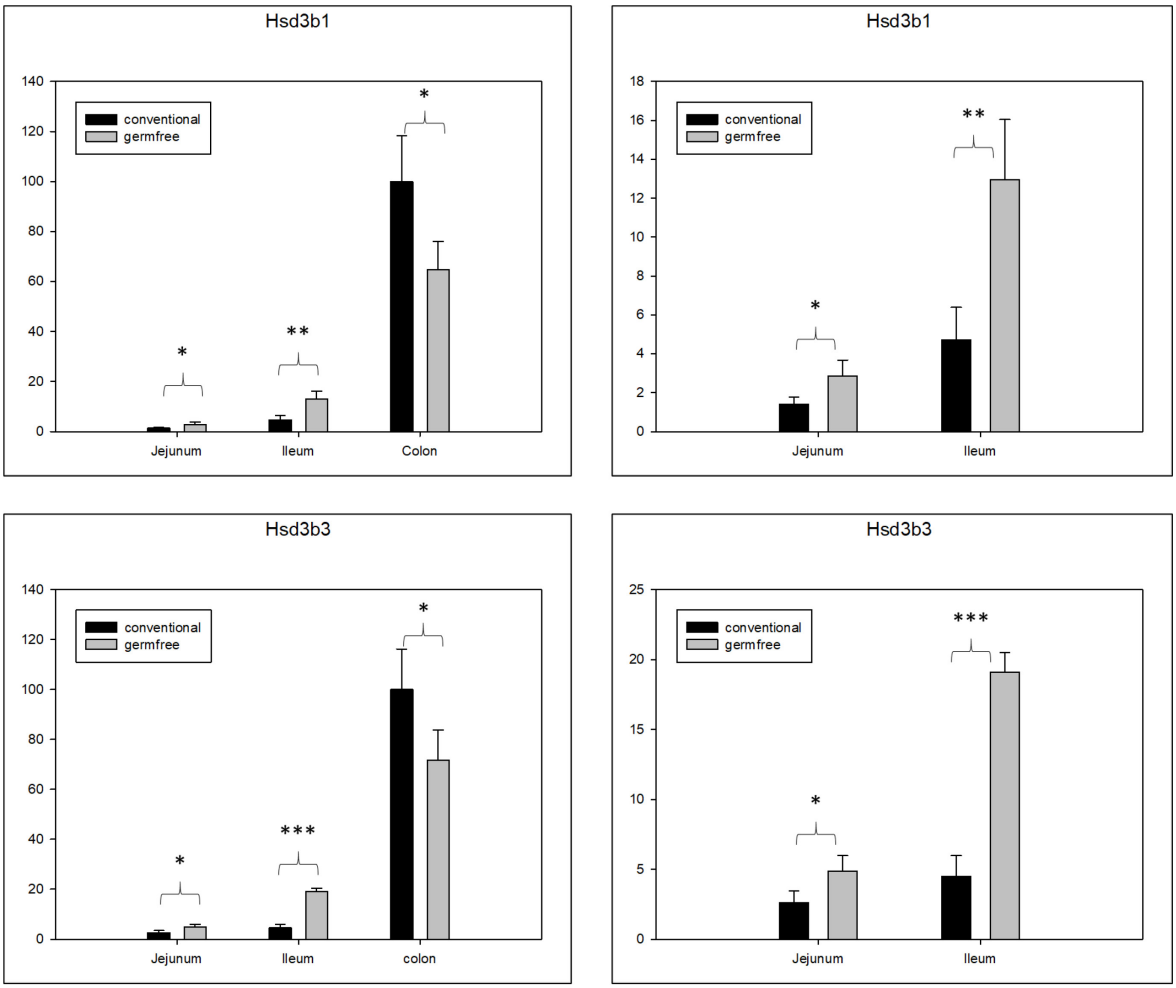


Figure 7 ***qPCR of HSD3B1 and HSD3B3 in the intestine of conventional and germfree mice. Results are displayed in % of conventional colon. The small intestine is additionally shown separately for better visibility of changes between conventional and germfree mice. n=5 mice for each condition. (* p<0,05; ** p<0,01; *** p<0,001).***

4.1.3 Non-steroidogenic LRH-1 and GR Target Genes

HSD3Bs have been reported to be regulated by LRH-1 in the mouse intestine. To further detect transcriptional effects of a possible ligand for LRH-1 in the gut microbiome, we additionally tested different non-steroidogenic LRH-1 target genes.

SHP is an atypical nuclear receptor that represses other transcription factors. SHP is not only a co-repressor for LRH-1, it has also been described as an LRH-1 target gene (117). Along the intestine of conventional mice SHP was expressed equally in all parts, comparable to LRH-1. Microbiome dependent expression showed similar patterns to the HSD3B genes. It showed trends to increased expression in the ileum of germfree mice and significantly decreased expression in the large intestine of germfree mice compared to conventional mice (Figure 8).

The LRH-1 target gene mouse fibroblast growth factor 15 (mFGF15) is equivalent to human FGF19. It is mainly expressed in the ileum and plays a role in bile acid metabolism (117). FGF15 showed a significantly decreased expression pattern in the ileum of germfree mice compared to conventional mice. Expression in the colon was at the detection limit (Figure 9).

Glucokinase (GCK) is involved in glucose metabolism and is mainly expressed in the pancreas and liver, where it is regulated by LRH-1 (115). GCK showed low levels of expression along the intestine, without significant differences in relation to the microbiome.

Since LRH-1 regulates steroidogenesis in the intestine, an agonist or antagonist ligand could affect glucocorticoid levels in the intestine. With the available samples we could not measure glucocorticoid levels itself, so we tested for effects of changes in glucocorticoid levels by analyzing the expression of the GR target gene Apolipoprotein A-IV (APOA4). APOA4 is mainly expressed in the intestine and is involved in lipid metabolism.
APOA4 showed significantly increased expression in the colon of germfree mice. Like FGF15 it had much higher levels of expression in the ileum compared to the colon (Figure 10).

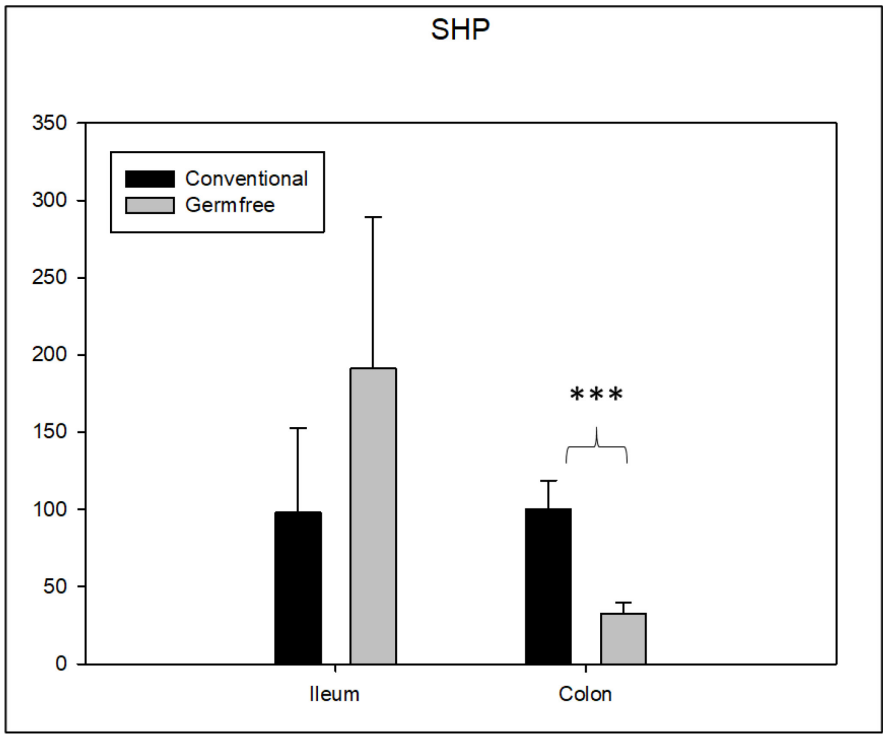


Figure 8 ***qPCR of SHP in ileum and colon of conventional and germfree mice. Results are displayed in % of conventional colon. n=5 mice for each condition. (*** p<0,001).***

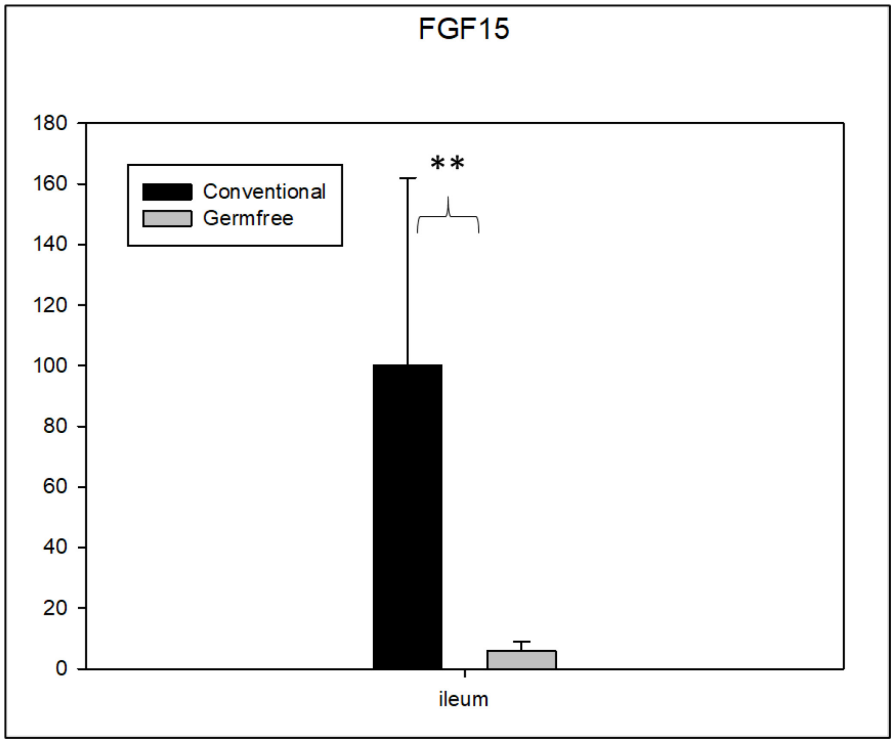


Figure 9 ***qPCR of FGF15 in the ileum of conventional and germfree mice. Results are displayed in % of conventional ileum. n=5 mice for each condition. (** p<0,01).***

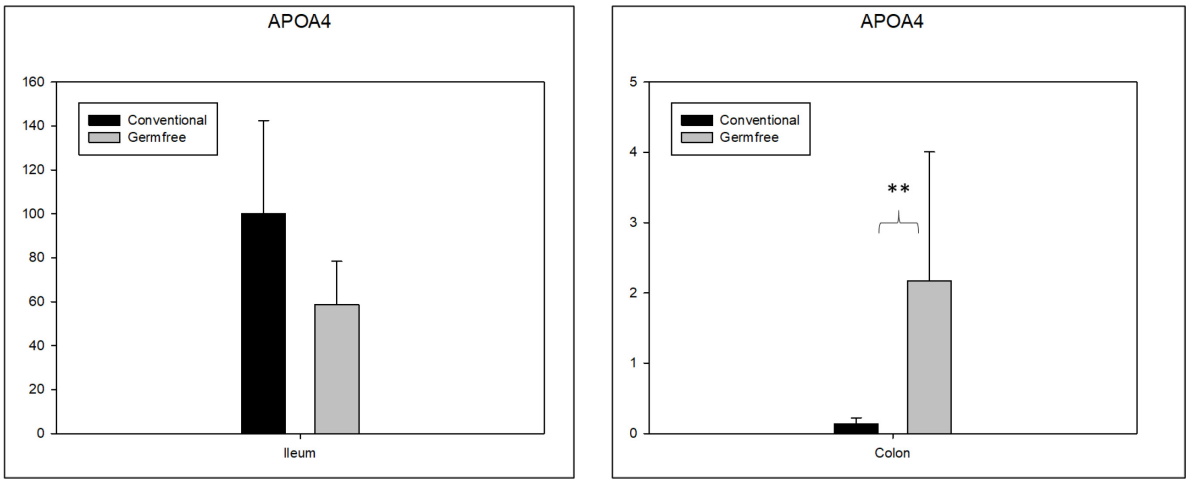


Figure 10 ***qPCR of APOA4 in ileum and colon of conventional and germfree mice. Results are displayed in % of conventional ileum and shown separately for better visibility. n=5 mice for each condition. (** p<0,01).***

4.2 Protein Levels of Steroidogenic Enzymes in the Intestine

We next determined protein expression of the most relevant steroidogenic enzymes CYP11A1, CYP11B1 and 3βHSD by Western Blot (WB). For establishing the antibodies, we used human and mouse adrenals for CYP11A1 and mouse adrenal glands for CYP11B1, since these tissues show high endogenous expression levels of the respective steroidogenic enzymes. The WBs to establish the antibodies are not shown here.

Western Blot for 3βHSD did not show a single distinct band for the enzyme. Presumably, the antibody bound to multiple isoforms. According to the datasheet the correct band is at 42 kD (Figure 11).

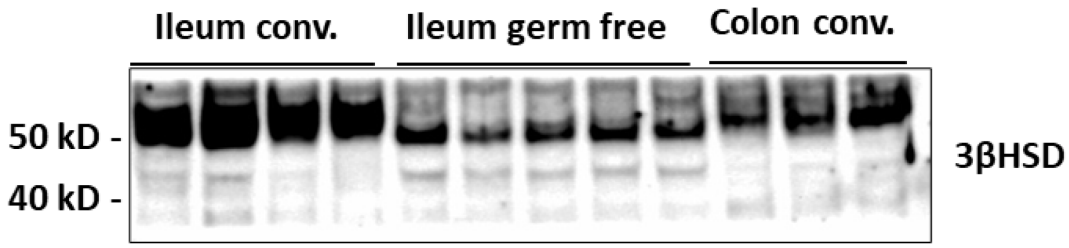
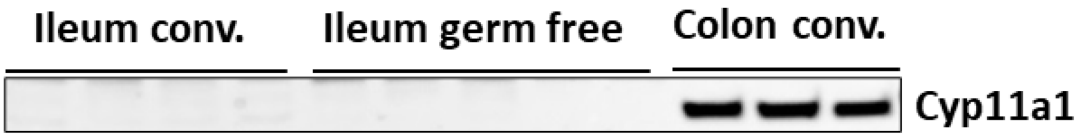


Figure 11 ***WB of 3βHSD in conventional ileum and colon and germfree ileum.***

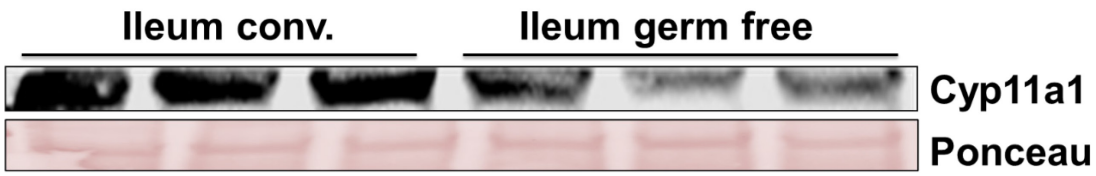
4.2.1 CYP11A1

Protein levels of the key steroidogenic enzyme CYP11A1 were considerably higher in the colon than in the ileum (Figure 12 A). After increasing protein load and antibody concentration in the ileum and decreasing these parameters in the colon, differing expression levels between germfree and conventional mice became more apparent. In both ileum and colon CYP11A1 was reduced in germfree mice. The decrease in expression was particularly noticeable in the ileum (Figure 12 B and C).

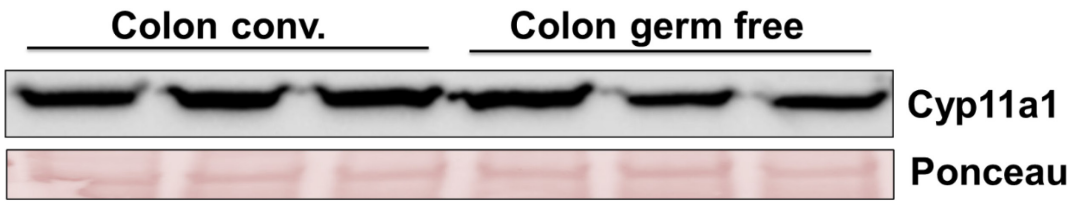
In line, immunofluorescence IF of CYP11A1 in the intestine of conventional and germfree mice showed the highest expression in the colon of conventional mice with a slight reduction in germ free mice. The ileum of germfree mice showed a striking reduction of CYP11A1 in IF (Figure 13).



A



B



C

Figure 12 ***WB of CYP11A1 in ileum and colon of conventional and germfree mice. A: 20 µg protein load, 1. antibody 1:1000 B: 45 µg protein load, 1. antibody 1:500 C: 10 µg protein load, 1. antibody 1:2000.***

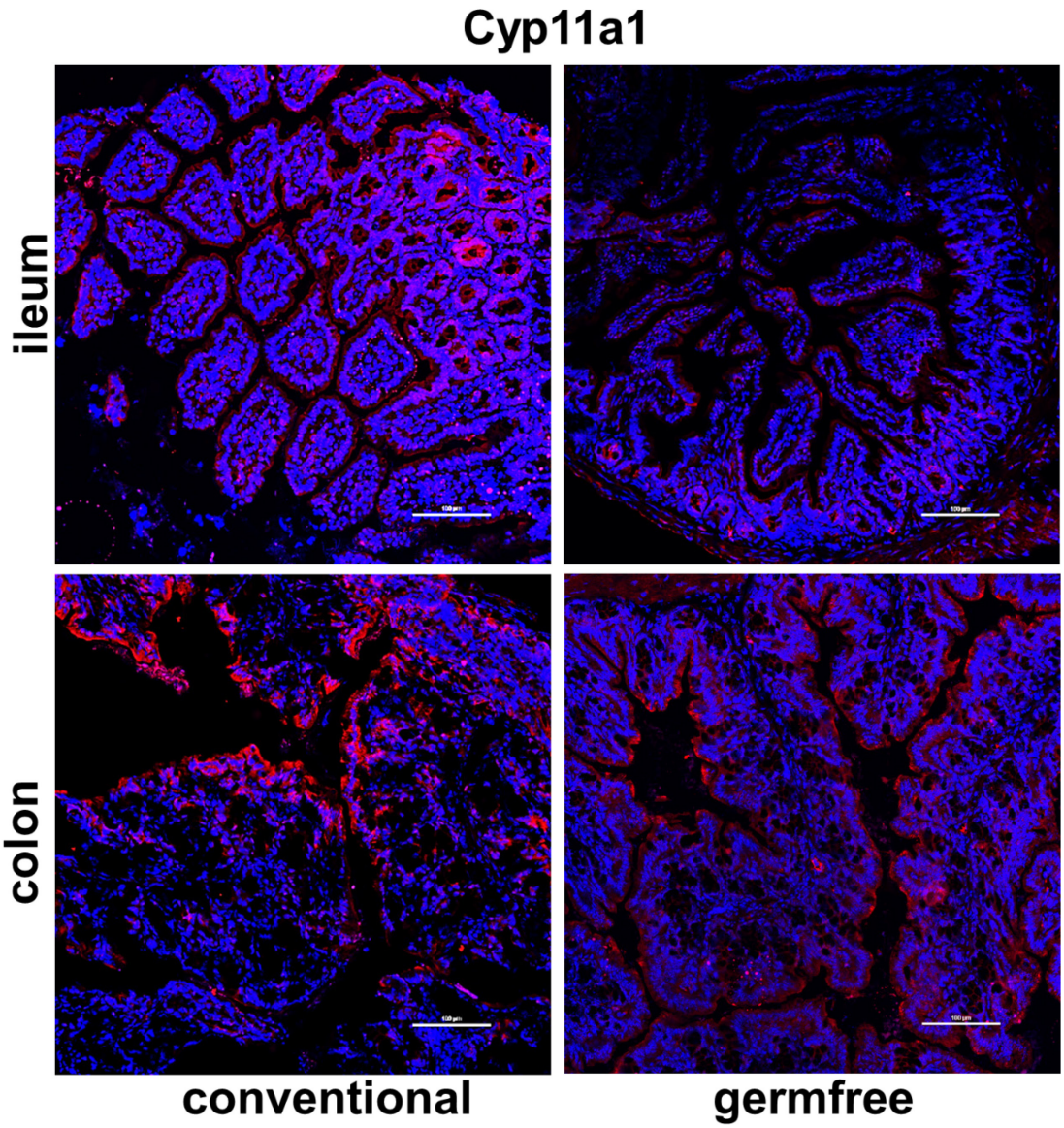
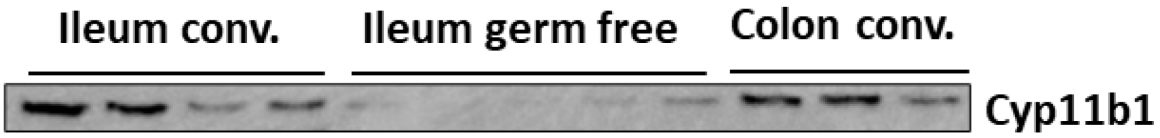


Figure 13 ***IF of CYP11A1 in ileum and colon of conventional and germfree mice.***

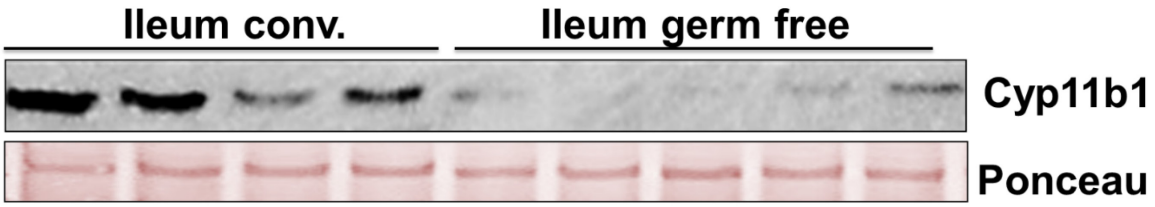
4.2.2 CYP11B1

WB for CYP11B1, responsible for the last step in glucocorticoid synthesis, had similar levels of expression along the intestine (Figure 14 A). Protein levels showed a strong decrease in colon and especially ileum of germfree mice (Figure 14 B and C).

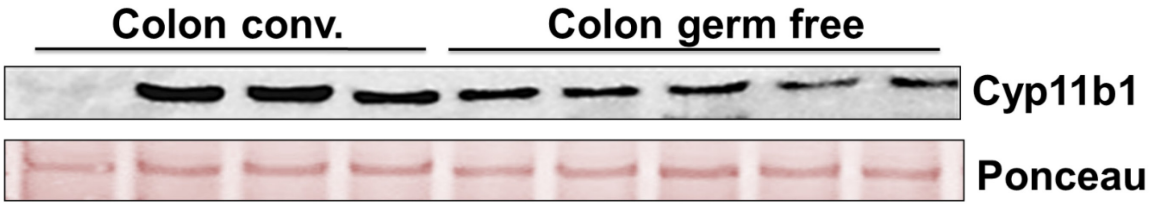
IF of CYP11B1 showed the highest levels of expression in the colon of conventional mice. Again, protein expression was reduced in germfree mice for both ileum and colon (Figure 15).



A



B



C

Figure 14 ***WB of CYP11B1 in ileum and colon of conventional and germfree mice A, B and C: 20 µg protein load, 1. antibody 1:1000.***

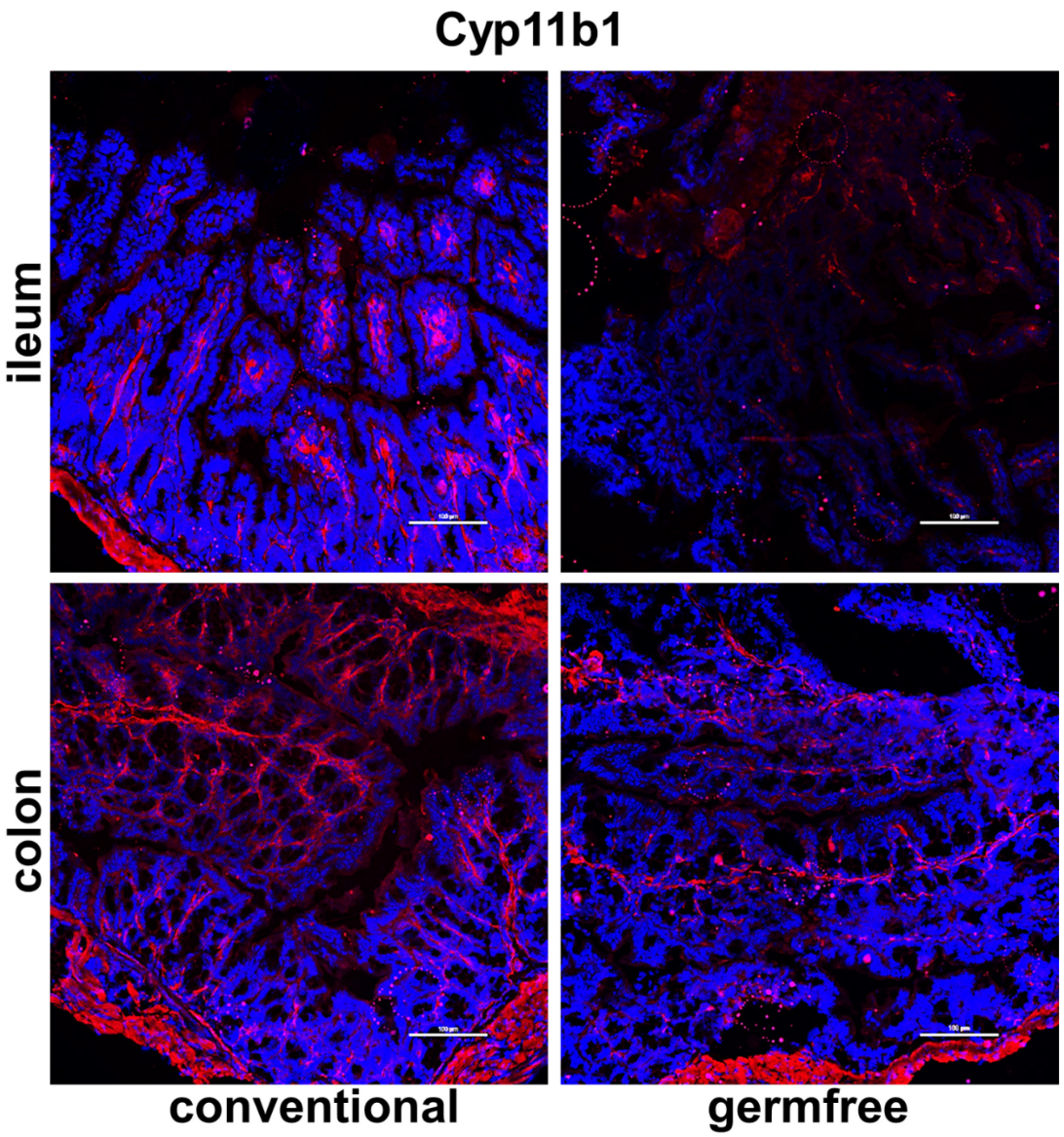


Figure 15 ***IF of CYP11B1 in ileum and colon of conventional and germfree mice.***

5 Discussion

The goal of this thesis was to determine if the gut microbiome has any effects on intestinal steroidogenesis. It is well known that intestinal steroidogenesis is regulated on transcriptional levels by the nuclear receptor LRH-1. Thus, microbiome dependent changes in expression of steroidogenic enzymes might provide hints to a potential endogenous ligand for LRH-1, generated by the gut microbiome. This could prospectively help explain the immunological mechanism of action in FMT and broaden treatment options for IBD patients.

We found that a lack of microbiome in germfree mice had a significant effect on expression of steroidogenic enzymes and non-steroidogenic LRH-1 target genes. Overall mRNA or protein expression of the key steroidogenic enzymes, which are all regulated by LRH-1, are significantly reduced in the colon of germfree mice. On mRNA levels HSD3B1 and HSD3B3 stand out with a notable increase of expression in the ileum of germfree mice. The overall expression of HSD3B1 and HSD3B3 is approximately 20 times higher in the colon, where most of the gut microbiome is located. We therefore interpret these effects as an overall reduction of HSD3B1 and HSD3B3. In line, FGF15 and SHP, which are both regulated by LRH-1, were also significantly reduced in germfree conditions.
Likewise, results of protein expression, determined by WB and IF, displayed a trend toward reduced expression in ileum and colon of germfree mice for CYP11A1 and CYP11B1. These results are suggestive of reduced GC production at least in the colon of germfree mice.

Unfortunately, we were not able to directly measure GC production in the intestinal tissue. Measuring GC synthesis in the tissue is rather complex and would require fresh material. Instead we used expression levels of the GR target gene APOA4 as a surrogate parameter. In contrast to our hypothesis, APOA4 had increased mRNA levels in the colon of germfree mice. This, however, does not necessarily mean that GC levels are in fact low. Future studies need to test more GR targets and determine immune cell infiltration.

We speculate that this mechanism might play a role in the pathogenesis of human IBD.
IBD patients reportedly have a less diverse microbiome and impaired intestinal steroidogenesis. A dysfunctional microbiome also produces a limited metabolome. The metabolome contains enzymes and proteins relevant for immune homeostasis and maintaining the TJB. Among this metabolome might exist a ligand for LRH-1 that is reduced

in the microbiome of IBD patients, thus leading to insufficient steroidogenesis and increased inflammation. FMT has been shown to induce remission in UC patients. Consequently, the next experimental step would be to determine expression levels of steroidogenic enzymes in IBD patients before and after FMT.

One explanation for the influence of the microbiome on steroidogenesis could be the generation of a ligand for LRH-1 by gut microbes. Such an LRH-1 ligand could provide a new treatment option for IBD, tackling the disease in two ways. The immunoregulatory effects could alleviate inflammation and regain homeostasis and with the impact of LRH-1 on cell renewal, a ligand could accelerate regeneration of damaged epithelium. However, the role of LRH-1 in tumorigenesis must be taken into consideration. LRH-1 is implicated in gastrointestinal tumors. Overstimulation of LRH-1 might amplify its impact on cell growth and entail an increased risk for gastrointestinal tumors in IBD patients, which are already at a greater risk for colon cancer. More targeted FMT that restores healthy conditions in a dysbiotic microbiome, might be a safe method to utilize the restorative functions of LRH-1, while minimizing the tumorigenic risks associated with LRH-1.

At base conditions steroidogenic enzymes are expressed at low levels in the intestine and only small amounts of GCs are produced. That made detection and analysis of enzyme expression difficult. Even with high concentrations of mRNA, CYP11A1 and CYP11B1 were at the limit of detection in qPCR. However, protein expression was more robust. The breakdown of mRNA might be faster than the breakdown of protein. The steroidogenic machine in the intestine primarily becomes active when faced with inflammatory stress. Effects of the microbiome on glucocorticoid synthesis might become more apparent in individuals who were immunologically challenged. Consequently, experiments in mice with models for inflammatory bowel disease might have shown more robust results.

Though we obtained notable results displaying effects of a lack of microbiome, there are some discrepancies we cannot explain at this time. One being HSD3B1 and HSD3B3 behaving in different ways in the ileum versus the colon. An explanation might be the differing composition and quantity of the microbiome between ileum and colon.

Out of all steroidogenic enzymes we looked at, CYP11B1 is the only one that is exclusively involved in glucocorticoid synthesis. The other enzymes, i.e. CYP11A1 and HDS3B1 are also involved in generation of local sex hormones, mainly estrogens. De-novo synthesis of estrogens in intestinal epithelial cells has been reported. Estrogens are also affected by changes. With a lack of microbiome conjugated estrogen does not get activated and reabsorbed, which might influence estrogen metabolism and synthesis.

We speculated that LRH-1 is the main regulator of intestinal steroidogenesis. Moreover, we hypothesized that activation of LRH-1 will depend on the gut microbiome. As a readout for LRH-1 activation, independent of steroidogenic genes, we showed induction of the LRH-1 target genes SHP and FGF15. Since a lack of microbiome affects a lot more than just glucocorticoid synthesis, it is challenging to separate effects from the reduced gut microbiome and other secondary effects. For example, bile acid metabolism is significantly changed in correlation with an altered microbiome. Several non-steroidogenic LRH-1 target genes are also regulated by FXR, which is influenced by bile acid composition. To separate effects of FXR and LRH-1 on their target gene expression, further experiments need to be conducted. One could be to perform experiments in LRH-1 knockout mice or do direct LRH-1 binding assays to promotors of genes.

In conclusion, we found that the gut microbiome significantly affects the expression of steroidogenic enzymes. It is established that intestinal steroidogenesis is regulated by LRH-1, but to determine if the observed effects of the microbiome are modulated by LRH-1, further experiments need to be performed. In addition, more studies will be necessary to see if specific strains of bacteria or parts of the metabolome in particular are relevant for intestinal steroidogenesis. Considering the sheer size of the microbiome and its metabolome, this might pose a challenge, but would improve comprehension on the way FMT works in IBD patients. FMT is a promising treatment option, but methods in preparation and application vary greatly. A more profound understanding in FMT's mechanism of action would help refine FMT methods and maybe even allow a more individualized approach, taking into consideration each patient's medical history and microbiome composition.

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