Department of Pharmacology and Toxicology

Faculty of Pharmacy in Hradec Králové

Charles University

Czech Republic

Bioinformatical aspects of nuclear receptors in pharmacotherapy

Doctoral thesis

Hradec Kralove 2022   Mgr. Josef Škoda

I hereby declare that this thesis is my original work that I solely composed by myself under the supervision of prof. PharmDr. Petr Pávek, Ph.D. All used literature and other sources are summarized in the list of references and properly cited. This work has not been submitted for any different or equal degree.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval samostatně pod vedením svého školitele prof. PharmDr. Petra Pávka, Ph.D. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

Josef Škoda

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ABSTRACT IN ENGLISH LANGUAGE

Candidate: Mgr. Josef Škoda

Supervisor: Prof. PharmDr. Petr Pávek, PhD.

Title of the doctoral thesis: Bioinformatical aspects of nuclear receptors in pharmacotherapy

The liver plays a central role in energy homeostasis via processing post-prandial excess energy into storage molecules and reusing stored energy via gluconeogenesis during the fasting period. This tight energy balance is maintained by a myriad of regulating processes. Dysfunction of metabolic control is a key event of severe diseases in today's population, starting with obesity, insulin resistance, and metabolic syndrome, proceeding to end-stage complications in type 2 diabetes, whole organ malfunctions, or even tumor diseases. Master players in metabolic regulation are nuclear receptors (NR) activated by endogenous stimuli or scavenging for nutritional or toxicological signals. NRs regulate gene transcription activation and therefore maintain liver metabolic plasticity.

In this thesis, modern molecular biology approaches were used to study ligands of NRs and the effects of their treatment. After the typical activation of a NR, hundreds of genes are regulated, which is beyond the ability to study with conventional biology methods. For this purpose, omics methods are an ideal solution. They are characterized by untargeted and comprehensive analysis of a large structural or functional group of molecules, e.g., transcriptome, proteome, or metabolome.

Constitutive androstane receptor (CAR) is a key regulator of the liver metabolism having a significant impact on a high-fat diet or leptin-deficient obese mice by improving insulin sensitivity and ameliorating fatty liver disease. However, most studies were performed with the mouse CAR ligand TCPOBOP, which exerts CAR off-target effects in CAR null mice. Therefore, we performed lipidomic and transcriptomic experiments after TCPOBOP treatment in wild-type and humanized CAR mice. Human CAR is not activated with TCPOBOP and thus maintains the basal physiological role of CAR. In humanized CAR mice, TCPOBOP elevated serum triglycerides, cholesterol, and glucose, increased liver triglycerides, and significantly altered liver transcriptome. On the other hand, TCPOBOP treatment and CAR activation in wild-type mice resulted in decreased serum triglycerides, cholesterol, and liver triglycerides. These divergent outcomes of TCPOBOP treatment between wild-type and humanized CAR

mice warrant the use of appropriate target-specific ligands in biological studies and suitable humanized animal or human 3D-organoid models in drug or chemical testing.

In the next part of this thesis, I studied experimental molecules and drugs reported as CAR ligands. I used *in silico* approaches for molecular docking, studied ligand-protein interaction, performed different gene reporter assays, and studied affected gene expression in primary human hepatocytes or humanized mouse models. I identified the drug diazepam as a weak-affinity CAR ligand interacting with the CAR ligand-binding cavity via hydrophobic interactions. Following experiments showed interaction of diazepam with CAR only in supra-physiologic concentrations in primary human hepatocytes and no activity in CAR humanized mice.

In summary, this work brought novel insights into testing NR ligands for their potential off-target and disruptive effects. Moreover, I characterized the impact of the widely used drug diazepam in liver regulations via CAR interaction.

ABSTRAKT V ČESKÉM JAZYCE

Kandidát: Mgr. Josef Škoda

Školitel: Prof. PharmDr. Petr Pávek, PhD.

Název doktorské práce: Bioinformatické aspekty nukleárních receptorů ve farmakoterapii

Játra zastávají centrální roli v energetické homeostáze, ukládají postprandiální přebytky energie do zásobních molekul a využívají zásobní energii pro glukoneogenezi během lačnění. Tato citlivá energetická rovnováha je udržována nesčetným množstvím regulačních procesů. Nefunkční kontrola metabolismu je klíčová podstata vzniku vážných onemocnění dnešní populace, začínající obezitou, insulinovou resistencí a metabolickým syndromem, pokračující ke komplikacím konečných stádií diabetu druhého typu, orgánového selhání nebo dokonce nádorovými onemocněními. Hlavní hráči regulace metabolismu jsou nukleární receptory (NR), které po aktivaci endogenními stimuly, nutričními nebo toxikologickými signály regulují transkripci a udržují metabolickou plasticitu jater.

V této práci jsme použili moderní metody molekulární biologie pro studium ligandů nukleárních receptorů a efektů jejich aplikace. Po klasické aktivaci NR jsou regulované stovky genů, studovat tyto děje je mimo možnosti klasických biologických metod. Pro tento účel jsou ideálním řešením *omics* metody, které jsou charakterizované necílenou a obsáhlou analýzou velké funkční nebo strukturní skupiny molekul, například transkriptomu, proteomu nebo metabolomu.

Konstitutivní androstanový receptor (CAR) je klíčový regulátor jaterního metabolismu se silným účinkem na metabolismus při vysoko-tukové dietě nebo u leptin deficientní obézní myši, kde aktivace CAR zlepšuje insulinovou rezistenci a zmírňuje jaterní steatózu. Nicméně, většina studií byla provedena s myším CAR ligandem TCPOBOP, který vykazuje také nepřímé efekty v geneticky modifikovaných myších bez CAR. Proto jsme provedli analýzu lipidomu a transkriptomu po aplikaci TCPOBOP na normálních (tzv. wild-type) a CAR humanizovaných myších, kde lidská varianta CAR není aktivována a udržuje fyziologickou funkci CAR. U humanizovaných myší TCPOBOP zvýšil triglyceridy, cholesterol a glukózu v séru, dále zvýšil hladinu jaterních triglyceridů a silně ovlivnil jaterní transkriptom. Podání TCPOBOP a aktivace CAR u normálních myší způsobily snížení triglyceridů a cholesterolu v séru, dále snížily hladinu jaterních triglyceridů. Rozdílná data na normálních a humanizovaných myších

potvrzují nutnost použití vhodných specifických ligandů v biologických studiích a užití vhodných humanizovaných zvířat, případně lidských 3D-organoidů pro testování léčiv a chemikálií.

V další části této práce jsem studoval ligandy CAR, jak experimentální látky, tak již užívaná léčiva ve farmakoterapii. Použil jsme *in silico* přístupy pro molekulární dokování, studoval interakci ligand-protein, provedl jsme různé gene reporter eseje a studoval ovlivnění exprese v primárních lidských hepatocytech nebo na humanizovaných myších. Identifikoval jsem diazepam jako ligand s nízkou afinitou k lidskému CAR, interagující s ligand-vázající kavitou CARu hydrofobními interakcemi. Následující experimenty ale ukázali aktivitu diazepamu k receptoru pouze v supra-fyziologických koncentracích na primárních lidských hepatocytech a žádnou aktivitu v myším CAR humanizovaném modelu.

Závěrem, tato práce ukázala nové přístupy v hodnocení ligandů NR pro jejich potenciální nepřímé a nežádoucí účinky. Dále jsem charakterizoval vliv užívaného léčiva diazepamu na regulace jaterních metabolických procesů přes nukleární receptor CAR.

Content

1. INTRODUCTION

In this thesis, I have summarized current insights into the regulation of liver metabolism by nuclear receptors. The introduction of this thesis is divided into chapters discussing the nuclear receptors, their study using the omics methods, and the importance of constitutive androstane receptor in the liver metabolic regulation.

1.1 Liver

The liver is the central metabolic organ that maintains the whole body's energetic homeostasis. The liver contains a blood volume of 25% of the cardiac output and utilizes up to 50 % of ingested glucose in the post-prandial period (Malarkey et al. 2005, Ferrannini et al. 1985). Hepatocytes, as the main hepatic parenchymal cells with metabolic activity, maintain tight energetic homeostasis by complex and precisely regulated cellular pathways and signaling. Additionally, the liver contains cholangiocytes (biliary epithelial cells), Kupffer cells (liver resident macrophages), stellate cells (retinoid storage cells and fibrogenesis), and endothelial cells, each specialized for the desired function (Vasconcellos et al. 2016).

As the central site for the synthesis of the carbohydrates and lipids in the body, the liver controls fluxes of plasmatic glucose and lipids during fasting and feeding periods. Thus, high functionality and fine regulation of liver metabolism are vital for our body. During the fasting period, the liver maintains normoglycemia via releasing glucose previously stored in glycogen (glycogenolysis) and via *de novo* glucose synthesis (gluconeogenesis), which is dependent on energy gained from fatty-acid (FA) β-oxidation. In the post-prandial period, nutrient intake with insulin, glucagon and other cytokines inhibits these processes and stimulate saving of energy in glycogen and *de novo* synthetized fatty acids stored in their neutral form, triglycerides (TG) (Jones 2016, Adeva-Andany et al. 2016, Alves-Bezerra and Cohen 2017). The malfunction of fine and complex control of the energy homeostasis leads to metabolic diseases, like metabolic syndrome, type 2 diabetes, and non-alcoholic fatty liver disease (NAFLD). Developed countries suffer from a worldwide pandemic of metabolic diseases, and there are strong epidemiologic links between insulin resistance, type 2 diabetes, and NAFLD; thus, the study of liver metabolism and its regulation is of high interest (Birkenfeld and Shulman 2014, Jones 2016).

1.1.1 Nuclear receptors

As metazoans evolved their bodies with a variety of compartmentalized function-divergent cells, tissues, and organs, the need for dynamic and adaptive signalization between them increased. Several tissues are fully dependent on energy supplementation in serving their specialized function and metabolic organs need to be informed about nutritional conditions in the body. Thus, complex neural and hormone signalization between the body compartments has evolved (Evans and Mangelsdorf 2014).

Functions of steroid and thyroid hormones were based on their physiological roles, but each of these chemicals is structurally distinct, and there was no presumption on common molecular mechanism. However, the initial biochemical experiments revealed the presence of intracellular receptors, which activate transcription of tissue and signal specific gene set upon ligand activation. This family of receptors was named nuclear receptors (NR). The isolation of the first complete NR cDNA and its use to test protein function helped to study receptor structure, its ligand-binding cavity with a variety of ligands, and characterization of receptor binding motif of DNA sequence which allows gene-specific expression. Interestingly, comparison of NR sequences revealed a highly conserved evolutionary template, including structural and functional features; DNA-binding, ligand-binding, and transactivation sites. For molecular endocrinology, the most important finding was the surprising discovery of dozens of evolutionarily related proteins. This evolutionary feature enabled organisms to recognize divergent endobiotic or xenobiotic molecules and specifically react to these stimuli. Because ligands of these receptors were unknown, it led to a newly characterized group of "orphan receptors". Today we know that the human genome encodes 48 NRs, and NRs modulate specific cellular pathways to endobiotic or xenobiotic stimuli, including different nutritional changes and toxicological or pharmacological responses. (Mangelsdorf et al. 1995, Evans and Mangelsdorf 2014, McKenna et al. 2009). This early work on the discovery of ligand-activated specific transcription factors resulted in firstly characterized real-time, plastic, and complex extracellular regulation.

The reality that there are several orphan NRs suggested the existence of unknown endogenous signaling molecules corresponding to a myriad of previously undiscovered pathways. Using the transfection assay with co-expression of studied receptor cDNA, and a receptor-responsive reporter gene with a highly specific and sensitive response of ligand-receptor interaction enabled "de-orphanization" of NRs (Evans and Mangelsdorf 2014). Co-expression assays of target receptors were instantly implemented in molecular biology and

pharmacology research to study NR ligands in micromolar concentrations as one of the most effective tools. The significance of this method is even more apparent compared to the classic approach of the first isolation of thyroid hormone from 3.5 tons of bovine thyroid gland (Kendall 1983). After 3 decades, recombinant NRs in gene reporter assays provided broad information on NR ligands acting in endobiotic signalization, response to xenobiotic stimuli, or even the use of NRs in pharmacotherapy.

Once activated, NRs directly regulate the transcription of genes that control various biological processes involved in metabolism, immunity, cell differentiation, and proliferation. Inactive NRs are typically located in cytoplasm anchored by their chaperones (e.g., HSP90). Once activated, they dimerize with their dimerization partner and translocate into the nucleus. In the nucleus, activated NRs create complexes with their coactivators that facilitate binding to specific DNA-binding motifs. Some NRs are in the passive state bound to their specific response elements and exert their repressive functions together with corepressor complexes NCoR and SMRT. After the activation, they dissociate corepressors and activate target genes with coactivator complexes (Sever and Glass 2013). Furthermore, some receptors can be activated indirectly via other signaling pathways or possess constitutive activity (Negishi et al. 2020). On all these levels, ligands compete for NRs with similar binding cavities, NRs compete for coactivators and corepressors. NRs have similar DNA-binding motifs and overlapping target gene sets; thus, even one NR can increase or decrease the metabolism of other NR ligands. These extremely complicated regulation processes between NRs are called the nuclear receptor crosstalk (De Bosscher et al. 2020). This complex mechanism delicately regulates whole-body development, highly specialized tissue sub-type differentiation, regulation of metabolic, endocrine, and exocrine processes, and immune reactions, where both robust and fine-tuning are needed.

In the liver, there are several master regulators of metabolism from the NR family. Peroxisome proliferator-activated receptors (PPARs), farnesoid X receptor (FXR), and liver X receptor (LXR) are primary sensors for energy intake via the portal vein (Figure 1). PPARs are major regulators of lipid metabolism in many tissues and are mainly involved in the liver-adipose tissue signaling axis. Behind the differences of PPAR subtypes and tissue specialization, they generally upregulate β-oxidation and cholesterol elimination. After feeding, FXR activated by bile acids promotes nutrient absorption, activates liver metabolism, and maintains bile-acid recirculation. LXR activated by oxysterols alters energy storage by activating triglyceride synthesis and distribution to peripheral tissues, as well as activating

reverse cholesterol transport to protect extrahepatic tissues against excess dietary cholesterol. Post-prandial hepatic activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR) promotes the clearance of toxic dietary metabolites, drugs, and endobiotics through phases I, II, and III of xenobiotic metabolism (Figure 1). Besides that, both PXR and CAR have functions in the regulation of hepatic energy metabolism (Evans and Mangelsdorf 2014, Cave et al. 2016).
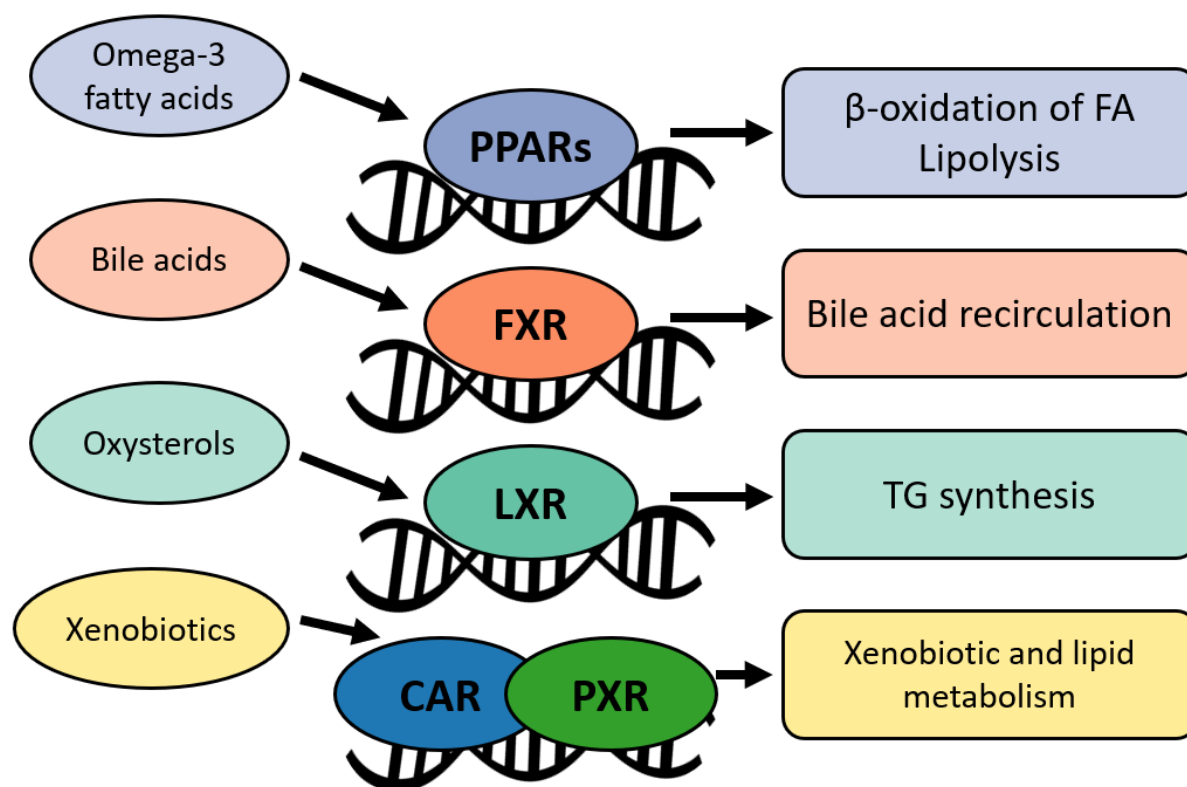

Figure 1. Key liver nuclear receptors in metabolism regulation. FA: fatty acids, TG: triglycerides (Evans and Mangelsdorf 2014).

2. OMICS APPROACHES IN THE STUDY OF LIVER METABOLIC REGULATION

The previous chapter about the complexity of liver metabolism and its regulation is the reason for the necessity of high-throughput analysis - traditional molecular biology methods aimed at single analyte, e.g., single mRNA transcript or protein level. Extensive scientific work has characterized many biological pathways, signaling cascades, and molecular mechanisms. However, since conventional biology proposed that biological organisms are much more complex than expected, the need for untargeted high-throughput methods was urgent. The geneticist Tom Roderick used the word "genomics" for the first time in 1986, 66 years after the

word "genome" was used for the first time. Subsequently, omics methods have been described as untargeted high-throughput approach of comprehensive characterization of a large group of molecules, grouped according to their structural or biological similarity (Aizat, Ismail and Noor 2018, Perakakis et al. 2018). Later, many more omics appeared, e.g., phenogenomics, metagenomics or pharmacogenomics. With the omic methods being more available, and as the analysis and interpretation become more advanced, the use of omics methods is more common every year, as evidenced by 874 "omic"-related publications registered in Scopus in 2021 (Figure 2).

Number of omics publications according to Scopus
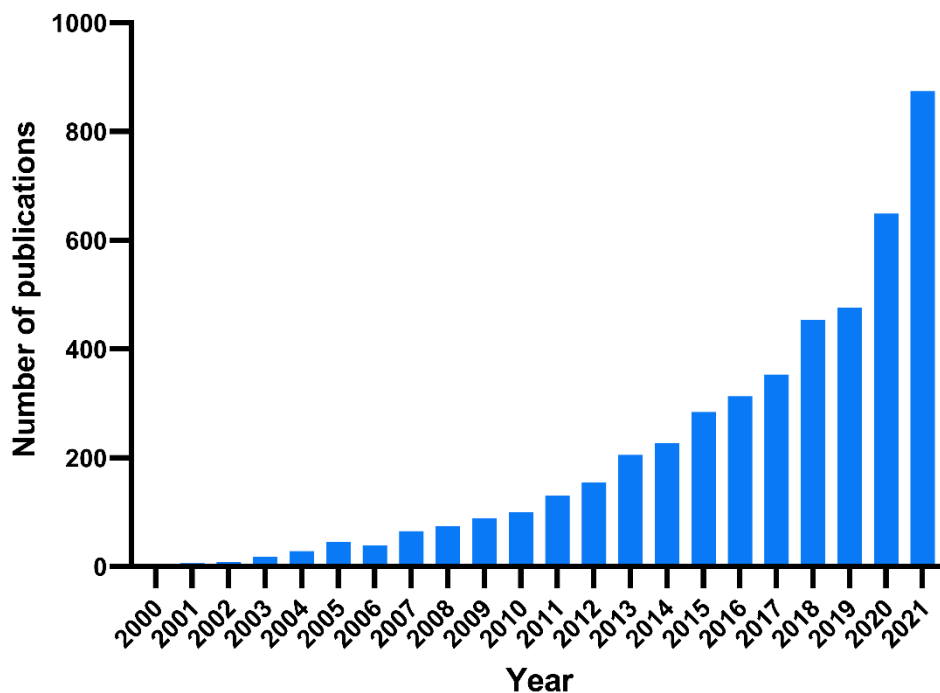
Figure 2. Scopus records including "omic" after 2000 (Scopus statistics).

Bioinformatics is a well-established part of biological sciences, characterized by using computer science for biological purposes. For molecular biology, bioinformatics was composed of studies of DNA and amino acid sequences, using them for artificial expression, predicting the three-dimensional shapes of proteins to determine their function or ligand-protein modelling interaction for molecular docking. However, the new millennium came with several new methods providing enormous amounts of data, characterizing the new "big data" period of science. High-throughput methods create so much data that data interpretation via directed

statistical analysis is impossible and typically behind the ability of one person (e.g., constructing statistical tests of control and experimental conditions for each analyzed feature) (Greene et al. 2014). In Human Genome Project, where the cloning method and Sanger sequencing were used, it took 11 years to sequence the human genome and the whole consortium was included (Gonzaga-Jauregui, Lupski and Gibbs 2012). Nowadays, high-throughput sequencing can provide us with a similar amount of information in one experiment.

2.1 Omics methods

Compared to the conventional approach, the opportunity to study biology with omics datasets including all analytes of studied level (genomics, transcriptomics, proteomics, metabolomics, etc.), has revolutionized the understanding of biological processes. Decreasing the time and cost to generate these datasets created interesting options and challenges for whole research teams, now including computational biologists and data analytics besides biologists. The use of omics data has been utilized in a broad range of research areas, including genotype-phenotype interactions, microbiology, analysis of microbiomes, nutrition science, and natural product discovery (Misra et al. 2018).

2.1.1   Next-generation sequencing platforms

After the era of Sanger sequencing, developed in 1977, new approaches have been developed. Microarrays have been introduced as the first method to study hundreds of analytes used for gene expression studies. The drawback of microarrays is that the hybridization sequences must be known and synthetized first, so de-novo transcript assembly is not possible. Nevertheless, optimized microarray assays are a powerful and cheap tool to study routinely performed studies, e.g., polymorphisms detection. Later, next-generation sequencing (NGS) was developed (Slatko, Gardner and Ausubel 2018).

NGS is generally based on a continuous readout of DNA sequence, e.g., sequencing by synthesis, while Sanger fluorescent terminating dideoxy terminators are replaced with the synthesis of complementary DNA and imaging of incorporated fluorescently labeled nucleotides (Figure 3). Another difference to Sanger sequencing is that only 50-300 base pair reads are sequenced with a higher error rate. However, NGS relies on high sequence coverage ("massively parallel sequencing") of millions to billions of short DNA sequence reads to obtain

an accurate sequence based on alignment to comparative sequence (genome). NGS platforms enable to work with low input samples and quantify these reads; thus, readout and quantification of sequences are possible using only one method. Most technologies utilize methods where DNA molecules are anchored to the solid substrate or separated into millions of separate wells. Subsequently, PCR amplification occurs, followed by the synthesis of complementary DNA with labeled nucleotides. Sequencing run usually takes hours to a day depending on throughput and generates up to 10^{10} reads (Slatko et al. 2018, https://www.illumina.com/systems/sequencing-platforms.html 2022).
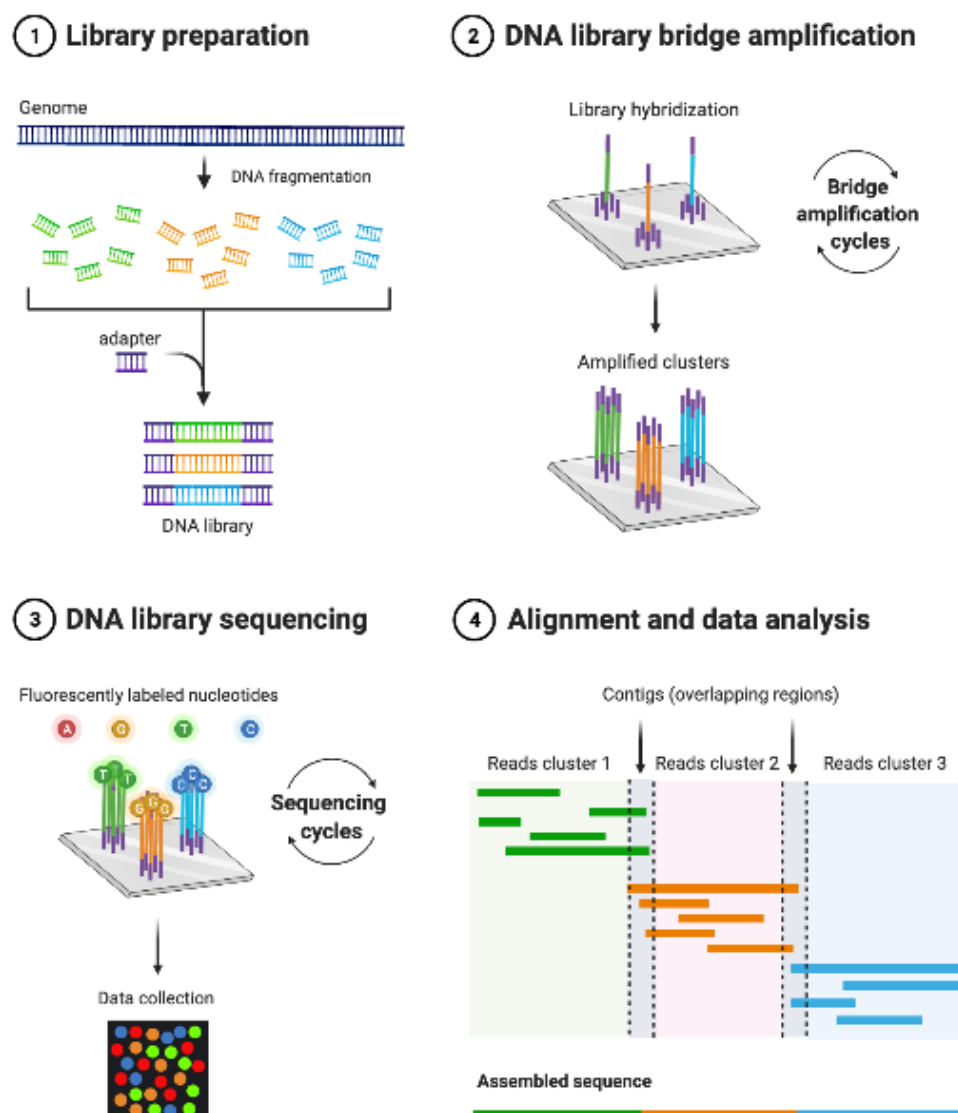

Figure 3. Illumina next-generation sequencing (Created with BioRender.com).

The first NGS platform was the 454 pyrosequencing, using the detection of pyrophosphate, a by-product of nucleotide incorporation. The whole readout happens on bead-tethered DNA 400-1000 bp sequences in picolitre chambers, where pyrophosphate release is measured. Although the method was later discontinued, it was commonly used for genomic sequencing because of long reads in relatively high-throughput (van Dijk et al. 2014).

Ion Torrent technology is based on complementary DNA synthesis on a semiconductor chip. When the correct nucleotide is incorporated, hydrogen releases and pH change is measured as a voltage change. Thus, sequential flooding of the chamber by only one nucleotide and voltage spikes captures the sequence; when two adjacent nucleotides are the same, voltage doubles. This technology is performed in millions of wells on semiconductor chips after PCR amplification on beads. The significant advantage of this method is that signal is recorded directly from the chip, and no camera with signal translation is needed. Thermo-Fisher now owns this technology with several platforms (Slatko et al. 2018, van Dijk et al. 2018).

So far, the major player, Illumina sequencing, is based on anchoring 50-250 bp DNA sequences with ligated adapters on both ends on a glass plate. Tethered sequences are subjected to repeated rounds of amplifications, known as the "bridge amplification" technique, which creates about 1000 copies of each DNA fragment. Complementary DNA is synthetized with fluorescently labeled nucleotides, and sequence is acquired as direct imaging. Illumina sequencing is the most used technique today, with almost all publications, including genomic, metagenomic, RNA, ChIP-seq methylome methods, being performed on this platform. Illumina provides different sequenators from iSeq100 to NovaSeq6000 with maximal throughput of 20 billion 250 bp reads, which create about 6000 Gb in one run (Slatko et al. 2018).

The drawback of before mentioned methods is that relatively short reads can result in misassembles, gaps in sequencing results, and repeating sequences longer than reads are hardly detectable (van Dijk et al. 2018). Also, PCR quantification and cluster amplification can cause biases. So, correct results require finding the "sweet spot" between amplifying enough to capture low abundant sequences and not quantifying too much to have too many reads occupied by overamplified sequences. Thus, "Third generation sequencing" has been established and aims to long reads, where during reading the original sequence, most of the original information is saved.

Firstly, Pacific Biosciences (PacBio) introduced SMRT (single molecule real-time) sequencing, which can sequence up to 50 kb long fragments. PacBio uses polymerase anchored

in the bottom of the well and fluorescently labeled nucleotides. The diameter of the well is smaller than the excitation light's wavelength, which causes it to fade out exponentially and illuminates only the very bottom of the special wells. Thus, only fluorescence of the nucleotides is captured, and this "interpulse duration" between the incorporation allows detection of nucleotide modifications. PacBio uses chips with millions of these wells; thus, high-speed parallel sequencing is possible. This platform uses fragments, which are ligated with adapters and circularized; therefore, multiple reading rounds are possible, with high reading precision acquired (>99.99%), or extremely long reads captured (up to 50 kb) (Slatko et al. 2018, van Dijk et al. 2018).

Finally, Oxford Nanopore Technologies introduced sequencers based on protein nanopores embedded in an electrically resistant polymeric membrane, and current changes tracked when the nucleotide of the DNA sequence passes through the nanopore. This technology has relatively high error rates, but this can be circumvented by a large number of sequenced molecules and by providing sequencers with a higher number of pores on the membrane. For further higher accuracy, harpin adapters were developed for double-stranded DNA sequencing, which allows sequencing of both strands (2D sequencing). Even though this platform does not provide a high throughput rate as Illumina or PacBio platform, they developed interesting platforms such as MinION (512 nanopores flow cell), which is ideal for expedition research. MinION is a portable instrument in size of a pocketknife with USB connectivity to a laptop, including the rapid sequencing kits that have a straightforward workout process and are ready for sequencing in 10 minutes (Slatko et al. 2018).

To catch the technology gap in long-read sequencing, Illumina developed SLR (synthetic long reads) technology. SLR partitions long DNA fragments into micro-wells or droplet emulsions where only a few fragments exist in one spot. Each partition is barcoded, classic short-read sequencing is performed, and the sequenced reads with the same barcoding are assembled locally as they come from one partition. 10X Genomics microfluidics system uses up to 10^6 barcodes; thus, it enables extreme high-throughput sequencing when connected to Illumina NovaSeq6000. This droplet barcoding is also used for single-cell sequencing (van Dijk et al. 2018).

As omics technologies opened a new universe of knowledge, single-cell approaches are the next leads in revolutionizing biology. Most of the current understanding of biology was gained with analysis of bulk tissue. However, mammals have at least 210 histologically diverse tissues, a number which would be many times higher when considering the functional

differences. Studying omics profiles of cell populations will lead to the discovery of new cell sub-types and reveal processes of physiologic or pathologic developments (Rauch and Mandrup 2016, Hwang, Lee and Bang 2018).

The most used single-cell sequencing platforms are 10X Genomics Chromium, DropSeq, and DropEach. These methods use microfluidics systems to tether and mark single cells with unique barcodes. The results from sequencing contain a number of transcripts for each cell further and enable cluster visualization of each specific cell population. Novel bioinformatic approaches also project information to multi-branched trajectories called pseudo-time analysis, which can display different events in a sample, e.g., differentiation or treatment effect. In single-cell approaches, RNA and ATAC-seq are effectively combined, showing that connecting multi- and single-cell omics approaches is an extremely powerful tool in modern science (Kulkarni et al. 2019, Andrews et al. 2021, Ranzoni et al. 2021).

2.1.2 Applications of NGS

The next chapter discusses the applications of NGS (Figure 4). The first application of deep sequencing was chromatin immunoprecipitation (ChIP)-seq, a technique based on immunoprecipitation of chromatin-associated protein (histone modifications, transcription factors) crosslinked to DNA followed by sequencing of fragments. By mapping these sequences to the genome, quantitative information is obtained on the locations occupied by transcription factors or epigenetic marks. ChIP-seq has proven extremely powerful by providing us with a revolutionary tool to study how transcription is regulated. ChIP-seq is limited by the need for highly specific antibodies, a high amount of input material (10^5 to 10^7 cells), and demanding sample preparation (Rauch and Mandrup 2016). Recently, a bead approach was developed, minimalizing the amount of input material. CUT&RUN is an even more advanced approach, where bead-anchored permeabilized unfixed cells/nuclei are incubated with the antibody of interest. Later, DNA sequences are obtained with protein A-Micrococcal nuclease (pA/MNase) fusion protein (Blecher-Gonen et al. 2013, Meers et al. 2019). Where crosslinking and fragmentation are demanding and can cause artifacts in the classic ChIP-seq, the CUT&RUN protocol is fast; nonetheless, it requires even more optimization in cell permeabilization and optimal MNase reaction. These advanced approaches are promising in studying TF binding without artificial noises.
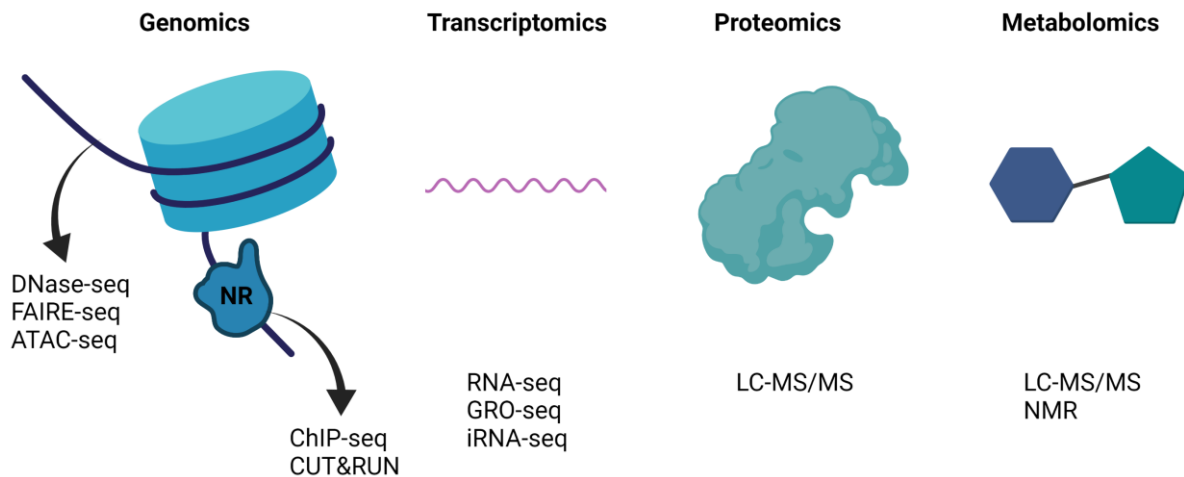
Figure 4. NGS applications (Created with BioRender.com).

Promoter, enhancer, and coding genome regions are actively released from nucleosomes by remodeling complexes and determining these nucleosome-depleted regions provides information about active regions in the genome. A map of these regions can be obtained by DNase I digestion of isolated nuclei followed by high-throughput sequencing (DNase-seq). Limited DNase I digests only small fragments from accessible regions. Appropriate mapping to the genome and sufficient sequencing depth can show precisely where TFs are binding in the genome. DNase I requires high cell numbers and precise titration of digestion (Rauch and Mandrup 2016). Next, the FAIRE-seq method uses crosslinking nucleosomes with DNA followed by sonication fragmenting and isolation of the fragments by phenol-chloroform extraction (Giresi et al. 2007). The recently developed method is an assay for transposase-accessible regions in chromatin (ATAC-seq), where transposase directly inserts sequencing adaptors into accessible regions (Buenrostro et al. 2013). These fragments are directly used for PCR amplification; thus, much fewer cell numbers are needed compared to DNase-seq and FAIRE-seq.

Today, high-throughput sequencing is also a key method in studying gene expression by RNA-seq. It has completely replaced the microarrays due to higher reproducibility, dynamic range, and the possibility to quantify before unknown sequences. RNA-seq relies on sequencing reverse-transcribed RNA depleted of rRNA (total RNA-seq) or mRNA (poly-A RNA-seq). Due to the high flexibility of the method, there are many approaches to study, e.g., newly synthesized RNA. iRNA-seq pipeline allows the determination of newly synthesized RNA by specifically quantifying reads in introns in total-RNA seq data. Transcription rates can be directly assessed by ChIP-seq on RNA polymerase II (RNAPII-ChIP-seq) or by global on-run sequencing (GRO-

seq). In the case of GRO-seq, transcription is paused and then allowed to continue with labeled nucleotides *in vitro*. Thus, GRO-seq shows the activity of RNA polymerase II in selected timepoints (Rauch and Mandrup 2016, Core, Waterfall and Lis 2008).

2.1.3 Proteomics

After NGS origin, a similar need for protein omics methods rose, and parallel methods were developed. The necessity of proteomics is important because mRNA levels do not always correspond with protein levels due to translational control, mRNA, or protein stability. Proteomics can also characterize protein phosphorylation, conformation, and consequently activity (Aslam et al. 2017, Kaur et al. 2018). Thus, proteomics approaches are used to quantify proteins, determine their localization and post-translational modification and study protein-protein interaction in different conditions.

Firstly, different high-throughput approaches of conventional protein analysis occurred. As DNA-hybridization microarrays were first used to generate transcriptomic data, microarray-based western blotting was developed and combined with conventional and two-dimensional gel separation and is still in active use (Treindl et al. 2016).

With soft-ionization methods, LC-MS/MS became the central method for protein analysis. Modern methods can identify over 10,000 proteins, almost connecting the gap to RNA-seq, where about 60,000 possible transcripts can be acquired in a human sample (Richards, Merrill and Coon 2015). Besides the conventional LC-MS, matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) is commonly used in protein detection. This approach was first used to detect bacterial infections, which are difficult to cultivate, and later used to analyze protein composition in complicated human or animal samples (Aslam et al. 2017). Furthermore, advances in LC-MS/MS technologies enabled the detection of complex protein samples (Huang et al. 2017). After MS characterization, there are approaches with quantitative steps that take advantage of label-based such as Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC), ^{18}O Stable Isotope Labeling, Isobaric Tagging for Relative and Absolute Quantitation (iTRAQ), and Tandem Mass Tags (TMT) (Misra et al. 2018). In summary, compared to NGS techniques, proteomics is experiencing fast growth and substantial advancement in instrument requirements, with chromatography coupled to mass-spectrometry methods being effectively and routinely used.

2.1.4 Metabolomics

Metabolomics is the next level in phenotype characterization within omics methods, focused on the analysis of small molecules (metabolites) within the sample. Although advanced NGS methods can distinguish genomic and transcriptomic events even on a spatial level, biologic events are altered due to protein function, a level in which previously mentioned methods are inadequate. Endobiotic or xenobiotic biologically active chemicals and pharmaceuticals affect the organism via direct interaction of the molecule with enzymes, e.g., inhibition, which alters in the first moment only on metabolite level.

Nuclear magnetic resonance (NMR), gas chromatography (GC-MS), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are methods to capture global metabolite composition and its possible changes. GC-MS, or more commonly LC-MS/MS combines the physical separation of metabolites with chromatography and highly sensitive/selective mass analysis. This combined platform allows to simultaneously identify hundreds of metabolites with high sensitivity and selectivity. The pitfall of MS analysis is that not all analytes are ionizable by using one type of instrument. Compared to MS, NMR spectroscopy is a highly reproducible analytical platform directly describing the structure of metabolites. Using two-dimensional ^1H-^1H correlation spectroscopy or ^1H-^{13}C heteronuclear single quantum coherence (HSQC) or total correlation spectroscopy (TOCSY), the NMR can analyze complex biological samples. The drawback of NMR is lower sensitivity compared to LC-MS/MS. Metabolomic methods branches into different structural groups of analytes based on the physical properties of metabolites and extraction. Conventionally, there are metabolomic analyses of small molecules studying basal metabolic events. Nonetheless, more specialized new methods were introduced with time, e.g., lipidomics explicitly aimed to extract and analyze lipids (Kim 2021, Cui, Lu and Lee 2018, Wishart 2019).

2.1.5 (Multi)-omics data analysis

Each method needs its specific preliminary data analysis to detect analyzed features and quantify them. NGS methods are characterized by aligning reads to genome or de novo assembly, while LC-MS/MS methods are distinguished with peak picking. All methods accommodate their specific normalization and relative or absolute quantification. After this process, the bioinformatician gets a large matrix of features, e.g., transcripts or metabolites (Lancaster et al. 2020). The statistical analysis can be divided into multivariate and univariate,

where the former analyzes multiple features at once, whereas the latter analyzes individual feature results separately (Misra et al. 2018).

Multivariate analysis is a powerful tool to visualize the similarity and clustering of all samples at once. Because of a high number of features analyzed in one experiment (from 10^2 to 10^5), dimension reduction is typically the first step in data interpretation. This means that every sample is a data point with so many dimensions as feature count. Subsequently, different techniques for data-dimensionality reduction are applied, resulting in fewer dimensions. Typically, the first dimensions show the most variability between the samples and enable visualization of this variability into a 2D graph. The most used unsupervised technique is principal component analysis (PCA); on the other hand, in single-cell data, it is typically tSNE or UMAP technique. Typically, the analysis starts with these techniques, and visualization can provide the most information about omics experiment – clustering of studied samples versus control, or plot showing batch effects (typically if sex, age, or other variables affected the data). Also, correlation analysis between samples is routinely performed as unsupervised multivariate methods, where Pearson correlation coefficients (PCC) and Spearman correlation coefficients (SCC) are most used.

For further visualization, there are different types of normalization. Data from omics experiments are distributed from zero or low abundance to several magnitude higher counts or concentrations. Normalizations are routinely used to alleviate problems with different expression or concentration levels, where z-score is the most common, which normalizes samples to their means. Also, log count transformation is used to skew data to an approximately normal distribution (Lancaster et al. 2020).

As the core figure of many omics studies, heatmaps are typically z-score values depicted after clustering. Hierarchical and k-means clustering are the most used clustering algorithms which compare differences in features between the samples. The results are typically upregulated or downregulated groups of features between the groups of samples (Lancaster et al. 2020).

One also wants to get the significantly regulated features, e.g., differentially expressed genes. With a lower count of features, a common Student's *t*-test later corrected for multiple testing can be used. Methods providing many features like RNA-seq have evolved their own statistical approaches, like the DESeq2 linear regression method (Love, Huber and Anders 2014). Typically, these techniques have built-in R packages and generate $\log_2$ fold-change and

false-discovery rate (FDR), which is *p*-value corrected for multiple testing. Thus, analysis can be easy-going and relatively fast for thousands of analyzed features.

With these data, the enrichment of functional categories can be analyzed. Nowadays this is routinely used almost only for NGS data. In these cases, the differentially expressed transcripts are used to perform the statistical analysis, and the results are shown as most enriched biological processes, e.g., lipid metabolism or mitotic cell cycle. This analysis allows easy interpretation of the study results. There are several used pathways databases, e.g., Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Lancaster et al. 2020). GO database has a curated list of genes included in each pathway, whereas KEGG has the categories even organized to pathway maps, and generation of pathway regulation is possible. KEGG is also developing databases for small molecules, enzymes, and more networks; however, pathway enrichment in metabolomics is still in its early stages of infancy.
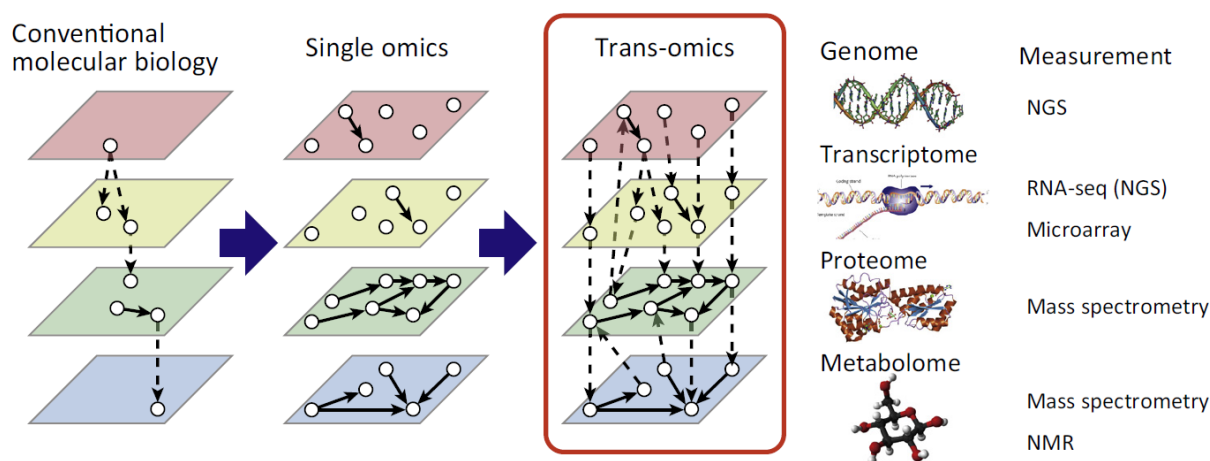
Figure 5. Multi-omics network across omics layers (Yugi et al. 2016).

Omics methods became a powerful method in research, but only one omics method does not show complete multi-layer information about the studied event. Choosing the wrong method for the study can result in no outcomes or misinterpretation of the secondary effect. When studying metabolic effects after inhibition of an enzyme, looking for epigenetic changes can be interesting but does not explain the mechanism of action and vice versa. Thus, novel approaches favor read-across multiple omics layers and elucidating molecular networks in three-dimensional relationships (Figure 5). This approach can use epigenomics to explain RNA-level regulations, proteomics to display post-translational modifications, and metabolomics to depict a functional snapshot of studied effects. This is essential since different

timepoints can display divergent reactions and adaptations to sequential reactions and regulatory events (Yugi et al. 2016). In recent years, revolutionary studies have revealed mechanisms behind years described physiological, histological, or morphological events, e.g., analysis of hematopoiesis (Ranzoni et al. 2021). Unfortunately, most multi-omics studies integrate only genomic and transcriptomic levels to date. This is mainly because metabolomics and its branches are a few years of development behind NGS methods. These methods need to be optimized for lower detection limits and mainly be included in automized bioinformatical analyses (Misra et al. 2018).

3. CONSTITUTIVE ANDROSTANE RECEPTOR IN METABOLIC REGULATION

Constitutive androstane receptor (CAR, NR1I3) is the key liver metabolism regulator with an important function in regulating both basal energy metabolism and inducible reaction to xenobiotic stimuli (Figure 6). CAR is a member of the steroid and thyroid receptors subfamily (NR1I), together with the pregnane X receptor (PXR, NR1I2) and the vitamin D receptor (VDR, NR1I1). Firstly, CAR was described as a ligand-activated xenobiotic sensor that induces transcription of genes included in xenobiotic metabolism, such as *CYP2B6* or *Cyp2b10* (Forman et al. 1998, Honkakoski et al. 1998). An interesting CAR feature is its basal (constitutive) activity in the absence of a ligand; thus, CAR ablation itself leads to disorders (Auerbach et al. 2003, Lukowicz et al. 2019). CAR is an orphan receptor with no observed endogenous ligand so far; thus, it was proposed as a xenobiotic sensor. There are considerable interspecies differences concerning ligands. Specifically, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO) is an experimental prototypic human CAR activator, while 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) is the mouse CAR prototypical activator (Maglich et al. 2003, Tzameli et al. 2000). Both ligands activate only species-specific CAR orthologue with no activity towards the other species orthologues – i.e., mice ligands do not activate human CAR and vice versa. Mouse and human CAR induce similar target gene sets, including metabolism-related genes; however, other mice and human CAR imprints in transcriptomes are different (Maglich et al. 2002, Niu et al. 2018). The most divergent and discussed CAR features are the promotion of liver proliferation and hepatocarcinogenesis, but these effects are conserved only to rodents (Yamada et al. 2020, Lake 2018). These species differences show the importance of using different models, e.g., humanized animal models or primary 3D spheroids, to cross-validate hypotheses connected to mouse CAR activation.

Importantly, CAR has been extensively studied as an endobiotic metabolism regulator in the last decade (Figure 6). Studies using wild-type (WT) and CAR null mice demonstrate that TCPOBOP CAR activation leads to reduced serum and hepatic triglyceride levels, improvement in glucose tolerance and insulin sensitivity in high-fat diet (HFD)-treated mice and in leptin-deficient *ob/ob* mice, in a CAR-dependent manner (Oliviero et al. 2020, Gao et al. 2009, Dong et al. 2009). Mouse CAR activation leads to better glucose tolerance and insulin sensitivity via downregulation of glucose-6-phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase (*Pck1*) in leptin-deficient mice (*ob/ob*). It also leads to lower hepatic lipid concentrations via downregulation of Sterol regulatory element-binding protein 1 (Srebp1), fatty-acid synthetase (Fasn), and Acyl-CoA desaturase 1 (Scd1), and more importantly, by increased *β*-oxidation (Dong et al. 2009). In another similar study, the authors treated mice for 5 weeks with a high-fat diet, where TCPOBOP treatment led to lower body weight and fat-to-body weight ratio and improved glucose tolerance (Dong et al. 2009). Previous studies also showed that TCPOBOP can protect the liver against cholestasis by upregulating cholesterol metabolizing enzymes (Cyp7b1, Cyp27a1, Cyp39a1) (Beilke et al. 2009, Guo et al. 2003). Lipid metabolism regulation through TCPOBOP-activated CAR has been suggested to be related to the modulation of PPARα and adiponectin signaling (Rezen et al. 2009).
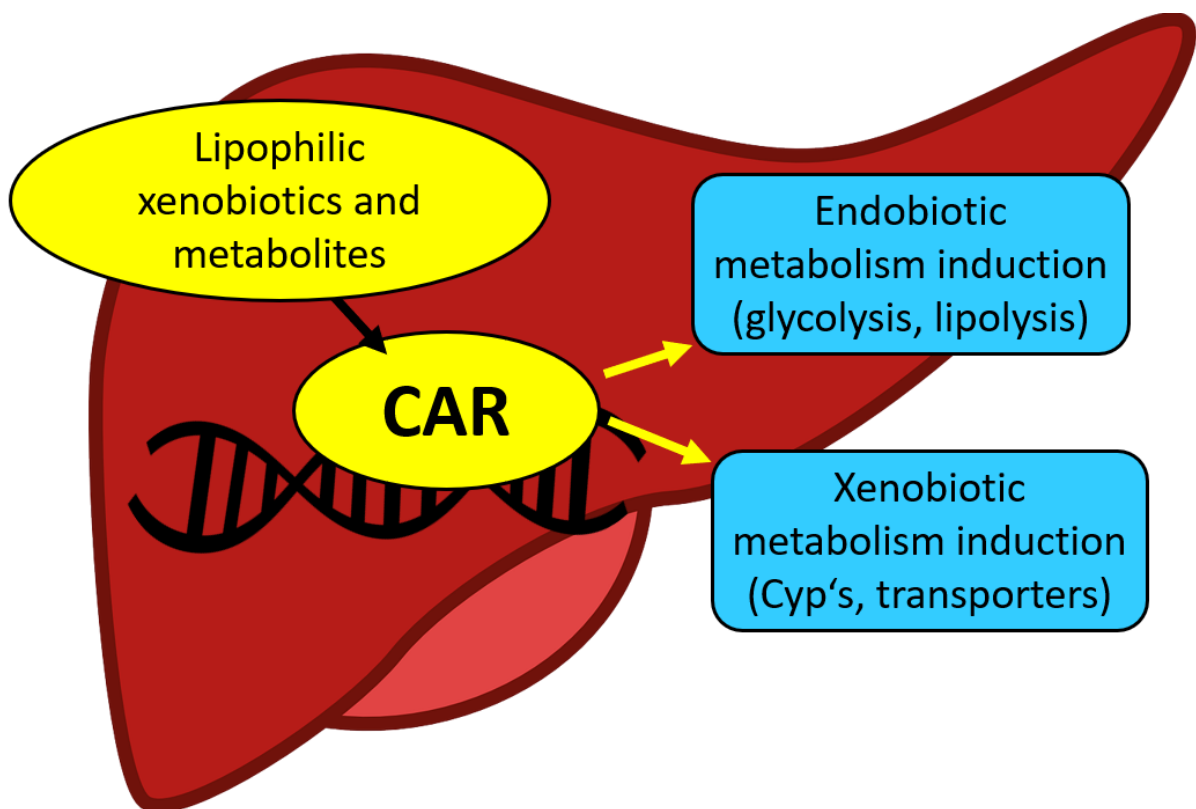

Figure 6. Effects of Constitutive androstane receptor (CAR) activation.

3.1 The role of CAR studied with omics approaches

Firstly, microarrays and soon after RNA-seq methods were used to describe ligand-activated CAR transcriptome. Studies on wild-type and CAR null models were performed using CAR direct activation with TCPOBOP and indirect activation by phenobarbital via EGF cascade. All these studies considered CAR activation as an event of metabolism induction and hepatic proliferation activation. Additionally, they compared CAR activation and its relative PXR, and RNA-seq was the key method to describe overlapping but distinct regulated transcriptomes (Cui and Klaassen 2016, Tojima et al. 2012, Luisier et al. 2014). Importantly, these models further showed gene-expression changes in CAR null models (Tojima et al. 2012, Park, Cheng and Cui 2016, Li et al. 2015). A recent report demonstrated that ablation of CAR itself causes metabolic disruption (Lukowicz et al. 2019). Further, array-based western blotting connected with laser capture microdissection revealed that upregulation of proliferation proteins is strictly localized to pericentral areas of the mouse livers with high CAR expression (Treindl et al. 2020).

Concerning human models, there is only one study published with transcriptome in human HepaRG cells using human CAR activator CITCO (Li et al. 2015). However, there are no data from primary human hepatocytes. Interestingly, there is one report comparing the transcriptomes of Hispanic to White donor livers, where Hispanics showed upregulation of CAR activity as well as upregulation of several CAR-target metabolism genes (Finkelstein et al. 2006). This publication also shows the importance of studies of different genotypes in the population and possible differences in metabolism-related pathologies or the effectiveness of pharmacotherapy. Unfortunately, the link between experimental knowledge on CAR and clinical outcomes has not been reported to date.

Interestingly, a recent study by Niu *et al.* reported transcriptional differences after injecting CAR null mice with mouse or human CAR vector. ChIP-seq revealed differences between the binding of receptors, where the mouse CAR showed the strongest binding to two hexamer half-sites DR4 structural motifs, while human CAR exhibited binding to only one hexamer. The presence or absence of the different ligands did not make the difference in the binding motif. This suggests that discovery of a selective acting agonist might be difficult for CAR. However, these small differences between CAR orthologs should be the clue to species-specific effects (Niu et al. 2018). Another publication has shown mouse CAR binding to lipid metabolism genes using ChIP-seq. In the study, CAR competition with HNF4α, PPARα, or FXR in enhancers was observed, which led to up- or downregulation of these genes (Tian et al.

2018). This metabolism-regulating network also showed an increase or decrease of liver chromatin accessibility in topologically-associated domains of genes regulated by TCPOBOP (Lodato, Rampersaud and Waxman 2018).

Further transcriptomic studies showed differences between male and female CAR activation. Specifically, while both sexes had similarly upregulated metabolism genes, the proliferative response was triggered only in males (Lodato et al. 2017). Also, using transcriptomic profiling, endocrine-disrupting chemicals (ECD) have been described as CAR activators (Lukowicz et al. 2018, Küblbeck, Niskanen and Honkakoski 2020a), and prototypical mouse CAR ligand, TCPOBOP, has been recently identified as a metabolism disruptor in humanized CAR mice (Skoda et al. 2021).

The metabolic approach revealed a significant shift in liver metabolism regarding CAR activity. Metabolism of glucose and amino acids was found to be stimulated, and gluconeogenesis decreased after CAR activation. This study has also shown enhanced serum fatty acids, ketone bodies and decreased sphingolipids, amino acids, and glucose (Chen et al. 2019). This study validates the significant potential of activated CAR in metabolic diseases and confirms the hypothesis of CAR beneficial metabolic effects acquired with conventional biology methods.

4. AIMS

The aims of this thesis were:

1. To study the complex *in vivo* effects of the mouse CAR ligand TCPOBOP using a multi-omics approach to reveal direct CAR and off-target effects, which could at least partially explain differences between divergent results obtained with different CAR activators and models.

2. Investigate small molecule pharmacological or xenobiotic CAR activators and evaluate their effects on liver metabolism using *in silico, in vitro,* and *in vivo* approaches.

5. LIST OF PUBLICATIONS RELATED TO THE DOCTORAL THESIS TOPIC

The aims of this work were fulfilled in the following publications in international peer-reviewed journals with impact factor:

1. **Skoda, J.,** K. Dohnalova, K. Chalupsky, A. Stahl, M. Templin, J. Maixnerova, S. Micuda, L. Grøntved, A. Braeuning & P. Pavek (2021) Off-target lipid metabolism disruption by the mouse constitutive androstane receptor ligand TCPOBOP in humanized mice. *Biochem Pharmacol,* 197**,** 114905. **(IF 2020 = 5.858, Q1)**

2. **Skoda, J.,** J. Dusek, M. Drastik, A. Stefela, K. Dohnalova, K. Chalupsky, T. Smutny, S. Micuda, S. Gerbal-Chaloin & P. Pavek (2020) Diazepam Promotes Translocation of Human Constitutive Androstane Receptor (CAR) via Direct Interaction with the Ligand-Binding Domain. *Cells,* 9. **(IF 2020 = 6.600, Q2)**

3. Dusek, J., **J. Skoda,** O. Holas, A. Horvatova, T. Smutny, L. Linhartova, P. Hirsova, O. Kucera, S. Micuda, A. Braeuning & P. Pavek (2019) Stilbene compound trans-3,4,5,4′-tetramethoxystilbene, a potential anticancer drug, regulates constitutive androstane receptor (Car) target genes, but does not possess proliferative activity in mouse liver. Toxicol Lett, 313, 1-10. **(IF 2019 = 3.569, Q1)**

4. Carazo, A., J. Dusek, O. Holas, **J. Skoda,** L. Hyrsova, T. Smutny, T. Soukup, M. Dosedel & P. Pavek (2018) Teriflunomide Is an Indirect Human Constitutive Androstane Receptor (CAR) Activator Interacting with Epidermal Growth Factor (EGF) Signaling. Front Pharmacol, 9, 993. **(IF 2018 = 3.845, Q1)**

6. AUTHOR'S CONTRIBUTION

My specific contribution to manuscripts associated with the doctoral thesis:

1. In the publication, I performed the following:
   a. Design of project together with P. Pávek
   b. Participated in animal work
   c. Performed RT-qPCR from liver samples
   d. Prepared samples for lipidomic, DigiWest and transcriptomic experiments
   e. Prepared library for RNA-seq
   f. Analysis of omics data
   g. Wrote manuscript and participated in the revisions of publication together with P. Pávek

2. In the publication, I performed the following:
   a. Design of experiments together with P. Pávek
   b. Performed TR-FRET
   c. Designed vectors of CAR with mutated ligand-binding cavity
   d. Cell culture of COS-1, HepG2, AML-12 and HepaRG cells, including the differentiation and treatment
   e. Performed all gene-reporter assays and RT-qPCR
   f. Transfected COS-1 cells with pEGFP-CAR vector and performed confocal microscopy
   g. Isolated liver samples and performed RT-qPCR from *in vivo* experiments
   h. Wrote draft of the manuscript together with P. Pávek

3. In the publication, I performed the following:
   a. Cell culture of HepG2 and AML-12 cells
   b. Performed gene-reporter assays
   c. EdU labeling of replicative DNA-synthesis
   d. Wrote parts of a manuscript concerning my experiments

4. In the publication, I performed the following:

    a. Cell culture of HepG2 and COS-1 cells

    b. Performed luciferase gene-reporter assays

    c. Transfected COS-1 cells with pEGFP-CAR vector and performed confocal microscopy

7. LIST OF PUBLICATIONS NOT RELATED TO THE DOCTORAL THESIS TOPIC

1. Stefela, A., M. Kaspar, M. Drastik, O. Holas, M. Hroch, T. Smutny, **J. Skoda**, M. Hutníková, A. V. Pandey, S. Micuda, E. Kudova & P. Pavek (2020) 3β-Isoobeticholic acid efficiently activates the farnesoid X receptor (FXR) due to its epimerization to 3α-epimer by hepatic metabolism. J Steroid Biochem Mol Biol, 202, 105702. **(IF 2020 = 4.294, Q2)**

2. Boltnarova, B., J. Kubackova, **J. Skoda,** A. Stefela, M. Smekalova, P. Svacinova, I. Pavkova, M. Dittrich, D. Scherman, J. Zbytovska, P. Pavek & O. Holas (2021) PLGA Based Nanospheres as a Potent Macrophage-Specific Drug Delivery System. Nanomaterials (Basel), 11. **(IF 2020 = 5.076, Q2)**

3. Jirkovská, A., G. Karabanovich, J. Kubeš, V. Skalická, I. Melnikova, J. Korábečný, T. Kučera, E. Jirkovský, L. Nováková, H. Bavlovič Piskáčková, **J. Škoda,** M. Štěrba, C. A. Austin, T. Šimůnek & J. Roh (2021) Structure-Activity Relationship Study of Dexrazoxane Analogues Reveals ICRF-193 as the Most Potent Bisdioxopiperazine against Anthracycline Toxicity to Cardiomyocytes Due to Its Strong Topoisomerase IIβ Interactions. J Med Chem, 64, 3997-4019. **(IF 2020 = 7.446, Q1)**

4. Pavek, P., J. Dusek, T. Smutny, L. Lochman, R. Kucera, **J. Skoda,** L. Smutna, R. Kamaraj, P. Soucek, R. Vrzal & Z. Dvorak (2022) Gene Expression Profiling of 1α,25(OH)$_2$D$_3$ Treatment in 2D/3D Human Hepatocyte Models Reveals CYP3A4 Induction but Minor Changes in Other Xenobiotic-Metabolizing Genes. Mol Nutr Food Res, e2200070. **(IF 2020 = 5.820, Q1)**

8. COMMENTARY ON THE PUBLISHED PAPERS RELATED TO THE THESIS

1. **Skoda, J.,** K. Dohnalova, K. Chalupsky, A. Stahl, M. Templin, J. Maixnerova, S. Micuda, L. Grøntved, A. Braeuning & P. Pavek (2021) Off-target lipid metabolism disruption by the mouse constitutive androstane receptor ligand TCPOBOP in humanized mice. *Biochem Pharmacol,* 197**,** 114905. **(IF 2020 = 5.858, Q1)**

CAR activation in various models has shown divergent results in the literature. CAR activation has beneficial effects in hypercaloric conditions, ameliorating fatty liver disease and improving insulin sensitivity; however, it promotes hepatic lipid accumulation in normal conditions. CAR activation in rodent models also triggers hepatic proliferation. Most of these results have been discovered with mouse CAR-specific ligand TCPOBOP in WT and CAR null mouse models. However, recent studies have shown some off-target effects of TCPOBOP in CAR null mice.

TCPOBOP is a polycyclic polyhalogenated aromatic compound with a high accumulation in adipose tissue, an extremely long biological half-life, and activity up to 140 days after exposure. Despite the high affinity of TCPOBOP to the mouse CAR, TCPOBOP may affect other biological targets and may interact with different pathways due to its persistence in the body.

To elucidate CAR-direct and off-target effects of TCPOBOP treatment, we compared WT and humanized CAR-PXR-CYP3A4/3A7 mice models. In the humanized model, human CAR is not activated by TCPOBOP and retains CAR basal activity. Because of CAR's high basal activity without ligand presence, CAR null models manifest metabolic disorders. For this purpose, we studied hepatic morphology, histology, hepatic lipid levels, and transcriptome.

After treatment, both WT and humanized CAR mice promoted higher liver weights, but humanized CAR mice showed hepatocyte hypertrophy without proliferative effects. Plasma levels of TG, cholesterol, and glucose were elevated in humanized CAR mice. Due to affected metabolism in humanized CAR mice after TCPOBOP treatment, we performed hepatic lipid profiling. The lipidomic analysis indicated significant TG accumulation in humanized CAR mice after short (3 hours) and long (48 hours) intervals. This model has also shown the accumulation of long-chain fully saturated lipids, often connected with NASH conditions in

literature. RNA-seq experiments of long interval-treated mice revealed deregulation of many genes involved in lipid metabolism in humanized CAR mice with affected PPAR, leptin, thyroid, and circadian-clock signaling. Interestingly, RT-qPCR analyses of affected genes from long interval RNA-seq study showed no significant regulation after short interval treatment in humanized mice. This indicates that liver lipid accumulation is an early event when the disruption of liver signaling is an adaptation to the initial metabolic changes. Compared to WT mice results, liver proliferation is conserved to the direct TCPOBOP-mediated CAR activation. However, all other metabolic effects were normalized or even reversed in WT mice with a decrease in plasmatic and liver lipid levels. Hypothetically, because CAR activation in WT mice can reverse metabolic disruption caused by TCPOBOP, CAR activation is highly beneficial in the case of metabolic diseases. The divergent effects of TCPOBOP in humanized CAR mice compared to WT mice also show the significance of appropriate ligands and models in testing pharmacological effects or metabolic disruption.

2. **Skoda, J.,** J. Dusek, M. Drastik, A. Stefela, K. Dohnalova, K. Chalupsky, T. Smutny, S. Micuda, S. Gerbal-Chaloin & P. Pavek (2020) Diazepam Promotes Translocation of Human Constitutive Androstane Receptor (CAR) via Direct Interaction with the Ligand-Binding Domain. *Cells,* 9. **(IF 2020 = 6.600, Q2)**

The aim of this work was to characterize the interaction of drug diazepam and CAR because of possible adverse effects on liver endobiotic and xenobiotic metabolism. In the past, diazepam was described as an indirect CAR agonist. Therefore, it is supposed that diazepam could affect the elimination of drugs metabolized by CAR target CYPs or modulate liver energy homeostasis.

We used a multilevel approach to study possible diazepam-CAR interactions. Firstly, we tested diazepam and its metabolites nordazepam, temazepam, and oxazepam in translocation assay. All molecules acted as CAR activators after occupying the CAR cavity and translocated the EGFP-labelled receptor into the nucleus. *In silico* docking approach proved that lipophilic diazepam and its metabolites exhibit similar affinities to the apolar CAR cavity. Benzodiazepines formed the hydrophobic contacts with Ile164, Leu206, Val232, and other prominent interactions with hydrophobic amino-acid residues of the CAR cavity. This was then tested in gene reporter experiments with CAR vectors with mutated ligand-binding cavities. Mutated vectors Ile164Ser, Leu206Ser, and Leu242Ser lost the inducibility by diazepam. Diazepam activity was also effectively inhibited by PK11195, the CAR inverse agonist acting in the ligand-binding cavity.

However, in a series of other experiments, including CAR3 variant gene reporter assay, CAR assembly assay, and TR-FRET experiment, only diazepam significantly induced CAR activity. Diazepam also induced CAR target gene CYP2B6 mRNA in HepaRG cells and primary hepatocytes, where induction was abolished by siRNA-CAR. However, its metabolites promoted no induction in these experiments. Thus, the more hydrophobic metabolites of diazepam showed lower affinity to CAR and were not able to activate CAR in physiological conditions. Next, we administered diazepam to humanized CAR-PXR-CYP3A4/3A7 mice model in 1 mg/kg dosing, which is approximately the maximum dosage in human medicine. Under these experimental conditions, diazepam had no activity towards CAR.

In summary, we characterized diazepam as a CAR ligand with low affinity that is rapidly lowered upon its metabolization. This study confirms diazepam safety in clinics regarding CAR

activation, and we can thus assume no adverse effects on drug-metabolizing enzymes and liver metabolism.

3. Dusek, J., **J. Skoda,** O. Holas, A. Horvatova, T. Smutny, L. Linhartova, P. Hirsova, O. Kucera, S. Micuda, A. Braeuning & P. Pavek (2019) Stilbene compound trans-3,4,5,4′-tetramethoxystilbene, a potential anticancer drug, regulates constitutive androstane receptor (Car) target genes, but does not possess proliferative activity in mouse liver. Toxicol Lett, 313, 1-10. **(IF 2019 = 3.569, Q1)**

In this work, we studied the possible experimental mouse CAR ligand, trans-3,4,5,4′-tetramethoxystilbene (TMS). Most of the publications studying CAR were performed with TCPOBOP, a polycyclic halogenated compound with extensive lipid accumulation, or the indirect CAR activator phenobarbital interacting with EGF cascade. Thus, there is a need for appropriate CAR experimental ligands with high and specific CAR affinity and good pharmacokinetics.

We used gene-reporter luciferase assays, where TMS was identified as the direct mouse CAR ligand with no activity to PXR. *In vivo* experiments showed that TMS promoted induction of drug metabolism (*Cyp2b10, Cyp2c29, Cyp2c55, Akr1b7,* and *Ugt2b34* genes) and downregulated genes involved in glucose and lipid metabolism (*Pck1, G6pc, Fasn, Scd1, Acaca,* and *Srebf1* genes). Interestingly, TMS did not promote CAR proliferative response, which was studied by gene expression, immunohistochemistry, Caspase 3/7, and EdU incorporation assays.

We found TMS as a direct CAR agonist suitable for studies on the role of CAR in hepatic metabolism. However, the different profile and mechanisms of TMS effects after CAR activation compared to prototypical ligands should be further elucidated. For example, by using a possible selective-acting derivative of TMS that has not been described for CAR activation so far.

4. Carazo, A., J. Dusek, O. Holas, **J. Skoda,** L. Hyrsova, T. Smutny, T. Soukup, M. Dosedel & P. Pavek (2018) Teriflunomide Is an Indirect Human Constitutive Androstane Receptor (CAR) Activator Interacting with Epidermal Growth Factor (EGF) Signaling. Front Pharmacol, 9, 993. **(IF 2018 = 3.845, Q1)**

In this study, we characterized the interactions of leflunomide (LEF) and its active metabolite teriflunomide (TER) with CAR. Both drugs upregulate mRNA expression of *CYP2B6*, CAR target gene. CAR has two possible ways of activation, direct ligand-dependent activation, and indirect activation via inhibition of EGF signaling and subsequent CAR dephosphorylation. This mechanistic study aimed to reveal the mechanism of *CYP2B6* induction after LEF or TER treatment.

Using HepaRG cells and primary human hepatocytes, both LEF and TER were found to upregulate *CYP2B6* mRNA and induce CYP2B6 and CYP4A4 protein activities. Interestingly, luciferase gene reporter and assembly assays for testing ligands of CAR, sensitive indicators of CAR direct activation, were not induced by LEF nor TER. In the EGFP-CAR translocation assay, these drugs also had no activity on the CAR vector.

Because TER has been described as an EGF inhibitor, indirect CAR activation was proposed and confirmed by our results, where TER significantly inhibited EGF phosphorylation and its downstream target Elk1. Both LEF and TER also activated the glucocorticoid receptor, which upregulated CAR expression. Thus, the proposed mechanisms of upregulation of CAR target genes by LEF and TER include the interaction with EGF signaling and glucocorticoid receptor activation.

9. DISCUSSION

Conventional biology has described many pathways, but data from first omics experiments have shown that biological regulatory networks are more complex by at least one magnitude than expected. ChIP-seq experiments surprisingly showed about 10 000-25 000 binding sites for a single receptor within 250-1000 genes (Evans and Mangelsdorf 2014). These revolutionary results broke the classic consensus of promotor being the only genomic spot of gene expression regulation. NGS methods helped find enhancers function in expression regulations, revealed regulating elements extremely distant from the regulated genes, or discovered the complex and fine-tuning in gene expression regulation (Pennacchio et al. 2013). Considering the power of high-throughput and omics methods, they should now be included in every experiment focused on NRs functions.

In my thesis, I focused on the study of CAR with its impact on liver metabolism and the properties of selected CAR ligands in experimental biology or pharmacotherapy. I thoroughly characterized the murine CAR ligand TCPOBOP on transcriptomic and lipidomic levels and described its CAR off-target effects in a humanized CAR mouse model. In the next part of my work, I studied potential ligands of CAR used in pharmacotherapy, considering their possible adverse effects. We also described a new mouse CAR ligand, which is derivative of stilbene and has different *in vivo* effects compared to the prototypical CAR activation.

For NRs and pharmacotherapy, it is necessary to describe all beneficial or pathologic effects of NR activation thoroughly. Additionally, the ligands must be fully characterized with high specificity to desired NRs. Today, PPAR ligands are irreplaceable in the therapy of metabolic diseases, but several had to be discontinued in the clinic because of hepatic or cardiac toxicities (Nanjan et al. 2018). NRs are also studied as a possible adverse outcome pathway in sensing chemical pollutants. CAR has already been identified as a possible sensor of xenobiotics with its lipophilic and promiscuity ligand-binding cavity, whereby chronic activation can lead to metabolic disruption (Küblbeck et al. 2020b, Küblbeck et al. 2020a).

Most *in vivo* studies describing CAR have been performed with the prototypical mouse CAR ligand TCPOBOP. Because TCPOBOP displays some off-target effects in the CAR null model (Maglich et al. 2002, Tojima et al. 2012, Park et al. 2016), the real mechanism behind direct TCPOBOP-CAR and off-target effects should be studied. Our study has shown significant disruption of liver lipid metabolism by TCPOBOP independently of CAR activation.

TCPOBOP treatment promoted liver hypertrophy, elevation of plasma TG, cholesterol, and glucose in humanized CAR mice without CAR activation. Lipidome was strongly affected upon 3-hour treatment with an increase in TG. Additionally, 692 differentially expressed genes were identified in humanized CAR mice, including PPAR, leptin, thyroid, and circadian clock pathways genes. On the contrary, CAR activation in WT mice effectively reversed TG accumulation with even lowering effects on plasma TG and cholesterol (Skoda et al. 2021). Therefore, we characterized TCPOBOP as a lipid metabolism disruptor in the non-activated CAR mouse model. On the other side, CAR activation proved a highly beneficial way to fight dyslipidemia, showing the urgent need for novel human CAR activators appropriate for studies of human CAR activation effects.

With the perspectives described in this thesis, the study of TCPOBOP effects in humanized CAR mice is an excellent example of the advantages and disadvantages of omics methods. Transcriptomic response to 48 hours treatment with TCPOBOP in humanized CAR mice showed massive gene expression changes with several regulating pathways affected and upregulated liver lipid catabolism gene sets. However, this data did not correspond with elevated serum TG and cholesterol. Thus, it raised the hypothesis that this complex transcriptome regulation can be an adaptation to lipid accumulation, which appeared in shorter, 3-hour intervals. This hypothesis was confirmed on a smaller gene set with RT-qPCR analysis performed at short treatment interval samples, where no significant mRNA regulation occurred. Thus, RNA-seq proved effective in studying late mRNA response to metabolic disruption and showed strong liver plasticity in reaction to xenobiotic stimuli. However, RNA-seq was not able to describe the initial events of TCPOBOP treatment. Lipidomic analysis revealed the time axis of initial significant lipid accumulation in a short interval, which triggered an adaptation mechanism metabolic insult of TCPOBOP. In summary, using the multi-omics approach proved an exceptionally effective tool in studying CAR off-target effects of TCPOBOP. This is consistent with the assumption of multi-omics as a core of modern NR molecular biology research.

Our group also studied potential CAR ligands with high specificity and appropriate pharmacokinetic properties. 3,4,5,4′-tetramethoxy-trans-stilbene (TMS) has been described as a protective agent against chemically-induced rat hepatocarcinogenesis (Cichocki et al. 2014). Using this molecule in studies of CAR-induced hepatocarcinogenesis, we found out that TMS is a mouse CAR agonist. *In vivo* studies showed no regulations in the expression of proliferative

and apoptotic genes after TMS treatment, but regulation of metabolic *Cyp2B10, Pck1, G6pc, Fasn, Scd1* and other genes as typical CAR activation response.

Next, we studied the drug diazepam described as an indirect CAR ligand (Li et al. 2009). Diazepam has also been characterized as rodent *Cyp2b10* (mouse CAR target CYP) inductor and liver tumorigenesis agent (Parkinson et al. 2006). Diazepam is metabolized by cytochromes P450 inducible by CAR; thus, diazepam administration could lead to back-loop induction of its own metabolism (Skoda et al. 2020). In this study, we used a systematic approach to study the possible interaction of diazepam with the CAR ligand-binding cavity and CAR activation. Using recombinant CAR protein in TR-FRET assay, fluorescently labeled CAR vector in translocation assay, and gene reporter assays, we identified diazepam as a CAR activator. Performing the mutagenesis of the CAR ligand-binding cavity with hydrophilic residues showed direct CAR activation by diazepam. Next, by evaluating diazepam metabolites nordazepam, temazepam and oxazepam, we showed their lower affinities to CAR. Further, we tested diazepam *in vivo* in the humanized CAR mice model in relevant doses to human medicine. In these experiments, diazepam showed no significant activation of CAR and no regulation of CAR-dependent metabolism and proliferation genes (Skoda et al. 2020). Using recombinant proteins specifically designed for CAR ligand-binding cavity studies, we mechanistically characterized diazepam as CAR ligand with its metabolites losing the affinity to CAR. We also confirmed the safety of diazepam *in vivo* with respect to metabolism regulation despite CAR activation by diazepam in artificial experimental conditions.

Next, we characterized leflunomide and teriflunomide as inductors of *CYP2B6* and suppressors of *G6Pase* and *PEPCK1* genes in primary human hepatocytes. This regulation is the characteristic of CAR-mediated xenobiotic metabolism induction and regulation of endogenous metabolism. Further, they also upregulated CAR mRNA. Employing gene reporters, TR-FRET, and pEGFP-CAR translocation assay, we found that both drugs do not interact directly with CAR but interfere with EGFR signaling, which activates CAR indirectly by phosphorylation (Carazo et al. 2018). Thus, we characterized for the first time a CAR activator used in the clinic, with activity in therapeutically relevant doses.

10. FUTURE PERSPECTIVES

Nuclear receptors are intensively studied as crucial regulators of metabolism in biology and as the core of modern pharmacotherapy facing the worldwide progress of metabolic diseases. The last two decades shifted research from single western blot gel manipulation and isolation of single receptors to an era of multidimensional omics data showing the high complexity of our body organization.

As omics methods evolve and become readily available, laboratories working on the regulation of biological processes will shift to using omics to depict the whole and complex processes behind NR regulation. We are at the beginning of an era when biological processes are explained via interpreting results from epigenetic to metabolite levels with all interactions and affected pathways visualized. Enhanced time and cost-effectiveness of omics methods will also be revolutionary for personalized medicine.

Taken together, using omics methods to design and characterize modulators of NRs activity is the modern clue for understanding healthy and diseased conditions, and the most significant advances are expected in the nearest future.

11. CONFERENCES ATTENDED

11.1 Oral presentations

02/2022 **12th Postgraduate and Postdoc Scientific Conference, Faculty of Pharmacy, Charles University**

"Off-target lipid metabolism disruption by the mouse constitutive androstane receptor ligand TCPOBOP in humanized mice"

01/2021 **11th Postgraduate and Postdoc Scientific Conference, Faculty of Pharmacy, Charles University**

"Murine constitutive androstane receptor (CAR) ligand TCPOBOP induced hepatomegaly in humanized CAR mice is independent of CAR activation"

01/2020 **10th Postgraduate and Postdoc Scientific Conference, Faculty of Pharmacy, Charles University**

"Characterization of transcriptome in humanized CAR mice regulated by model murine ligand TCPOBOP"

09/2019 **FEBS Advanced Lecture Course on Epigenomics, Nuclear Receptors and Disease, Island of Spetses, Greece**

"Constitutive androstane receptor (NR1I3, CAR) enhanced effect on endogenous metabolism: way through ligand optimization to ideal transcriptomic profile"

01/2019 **9th Postgraduate and Postdoc Scientific Conference, Faculty of Pharmacy, Charles University**

"Activation of human constitutive androstane receptor (CAR) by benzodiazepines without proliferative effect and liver tumorigenic effects in human cells"

11.2 Poster presentations

09/2019 **FEBS Advanced Lecture Course on Epigenomics, Nuclear Receptors and Disease, Island of Spetses, Greece**

"Constitutive androstane receptor (NR1I3, CAR) enhanced effect on endogenous metabolism: way through ligand optimization to ideal transcriptomic profile"

11/2018 **11th Nuclear Receptor Research Network (NR2N), Eindhoven**

"Discovery of a novel agonist of mouse Constitutive Androstane Receptor (CAR, NR1I3)"

12. ABBREVIATIONS

CAR	Constitutive androstane receptor
CYP	Cytochrome P450
FXR	Farnesoid X receptor
NASH	Non-alcoholic steatohepatitis
NGS	Next generation sequencing
LEF	Leflunomide
LXR	Liver X receptor
PPARs	Peroxisome proliferator-activated receptors
PXR	Pregnane X receptor
SLR	Synthetic long read
TER	Teriflunomide
TG	Triglycerides
WT	Wild-type
TMS	3,4,5,4´-tetramethoxy-trans-stilbene

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