Farnesoid X Receptor alters adipose tissue architecture in mice and limits storage capacity leading to metabolic derangements

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Abstract

The bile acid-activated nuclear receptor Farnesoid X receptor (FXR, NR1H4) has been implicated in the control of lipid and energy metabolism, but its role in fat tissue, where it is moderately expressed, is not understood. In view of the recent development of FXR-targeting therapeutics for treatment of human metabolic diseases, understanding the tissue-specific actions of FXR is essential. We show that transgenic mice expressing human FXR in adipose tissue (aP2-hFXR) have markedly enlarged adipocytes and show extensive extracellular matrix remodelling. Ageing and exposure to obesogenic conditions revealed a strongly limited capacity for adipose expansion and development of fibrosis in adipose tissues of aP2-hFXR transgenic mice. This was associated with impaired lipid storage capacity, leading to elevated plasma free fatty acids, ectopic fat deposition in liver and muscle as well as whole-body insulin resistance. These studies establish that adipose FXR is a determinant of adipose tissue architecture and contributes to whole-body lipid homeostasis.

Keywords: adipose, FXR, hypertrophy, hyperplasia, extracellular matrix, insulin resistance
**Introduction**

Bile acids are amphipathic steroids made from cholesterol in the liver that act as facilitators of intestinal fat absorption (1). It has become clear that this versatile group of molecules also regulates an array of physiological processes that are vital to hepatic, intestinal and metabolic health via activation of the nuclear receptor FXR (farnesoid X receptor, NR1H4) and the membrane-bound receptor TGR5 (GPBAR1) (1,2). Bile acids have also been shown to be able to activate the nuclear receptors vitamin D receptor and pregnane X receptor as well as sphingosine 1-phosphate receptor 2 (3,4,5). The combined properties of being a small molecule with potent physiological effects has driven efforts to utilize bile acids and/or their signalling systems for therapeutic applications (2). Obeticholic acid (OCA), a potent FXR agonist, has been approved for the treatment of primary biliary cholangitis (6). OCA was also shown to increase insulin sensitivity in type 2 diabetes patients as well as to improve markers of liver inflammation and fibrosis (7). Effectiveness of OCA treatment was also confirmed in a multicenter trial with non-cirrhotic, non-alcoholic steatohepatitis patients (8). Interestingly, despite hepatic improvements, this study also showed elevated insulin levels and increased values for Homeostatic Model Assessment of Insulin Resistance (HOMA index). Yet, the many implications of bile acid signalling pathways on energy metabolism has inspired the development of FXR agonists aiming at obesity-related diseases that are currently advancing into clinical trials (9,10,11,12).

FXR activation by bile acids (13,14,15) provides these natural detergents with a means to modulate their own synthesis in the liver and their transport within the enterohepatic circulation (1,16,17,18). Accordingly, FXR shows highest expression in the liver and intestine (19). Yet, FXR expression is not confined to these sites, but also occurs in several other organs and tissues including kidney, adrenal glands, vascular wall as well as adipose tissue. The physiological functions of FXR at these locations has largely remained elusive. In view of the recent developments towards the use of FXR agonists as therapeutic agents, it is of crucial importance to have full understanding of the (patho)physiological role of FXR in specific non-enterohepatic locations, including adipose tissue. This is of particular importance because obesity-associated diseases, such as type 2 diabetes and dyslipidemia, are characterized by adipose tissue dysfunction (20).
The capacity for dynamic remodeling of white adipose tissue (WAT) and its role in control of whole-body metabolism through crosstalk with other tissues and organs by metabolites (predominantly free fatty acids) and hormones (i.e., adipokines and lipokines), delineates the complexity of metabolic control (21). Whereas subcutaneous WAT is the sole site in the body that can accumulate lipids without detrimental consequences, expansion of visceral adipose depots and particularly ectopic lipid deposition in liver and muscle have major adverse health effects (22). Expansion by formation of new adipocytes (hyperplasia) is a hallmark of healthy WAT, whereas growth by enlargement of adipocyte size (i.e., hypertrophy) is associated with adverse metabolic outcomes (23).

A role for FXR in adipose tissue, where it is expressed at relatively low levels, was first proposed when FXR−/− mice were shown to exhibit small adipocytes and FXR was subsequently shown to promote adipocyte differentiation in vitro (24,25,26). These initial studies further revealed insulin resistance in adipose tissue of mice lacking FXR. Unexpectedly, whole body FXR-deficient mice were also shown to be resistant to the development of obesity when fed a high-fat diet or after crossbreeding with a genetic model of obesity, i.e. ob/ob mice. Both models showed improved glucose homeostasis as well as increased adipose insulin sensitivity (27). Interestingly, FXR expression in adipose tissues appeared to be decreased in mouse models of obesity (24). Yet, the direct contribution of adipocytic FXR in the development of these striking metabolic phenotypes has remained elusive.

Here, we describe the metabolic consequences of moderate (3-5 fold) overexpression of human FXR under the control of the aP2 (Fabp4) promoter in mice to directly address FXR function in adipose tissue. These studies show that, in line with the small adipocytes observed in FXR−/− mice (24), aP2-hFXR mice display a marked adipocyte hypertrophy. We examined the impact of FXR overexpression on WAT functioning under basal conditions as well as in response to metabolic stressors such as diet- and age-induced obesity. These studies establish that FXR is a key determinant of adipocyte size and fat tissue architecture as sustained FXR expression in adipose tissue limits its storage capacity, promotes adipose tissue fibrosis and ultimately drives ectopic lipid accumulation and development of whole-body insulin resistance.
Material & Methods

Transgene Construction and Generation of Transgenic aP2-hFXR Mice

The individual expression plasmids containing the mouse aP2 promoter in pBleuscript II SK(+) and the full-length open reading frame of the human FXRα2 in pCDNA3.1/TOPO were generous gifts from dr. R.E. Hammer (60) and dr. R. Mukherjee (61), respectively.

The 5.4 kbp fragment of aP2 promoter was excised from the pBleuscript II SK(+) using KpnI-SmaI restriction enzymes and ligated into the KpnI and SmaI sites of the pGEM-7Zf(+) vector (Promega, Madison, WI, USA). By PCR amplification SmaI-SmaI restriction sites were cloned onto the human FXRα2 fragment (using forward primer cccgggATGGGATCAAAAATGAATCT and reverse primer cccgggAGAATAGAATGACACCTACT). The resulting 1.9 kbp SmaI-SmaI human FXRα2 fragment was cloned into the pCRTMII Vector (Invitrogen, Breda, The Netherlands). After amplification, this fragment was excised, gel-purified and ligated to SmaI and SmaI sites of the pGEM-7Zf(+) containing the 5.4 kbp aP2 promoter fragment. The orientation of the human FXR gene was verified using gel-based restriction control and the plasmid was sequence verified for the presence of the correct fragment. Adipocyte-specific human-FXR transgenic mice (aP2-hFXR mice) were generated by microinjection of the construct in fertilized FVB/NHsd eggs. The clone with the highest human-FXR mRNA level was recovered. Wild type (wt) littermates were used as a control. aP2-hFXR mice were mated with whole-body Fxr-deficient (FXRβ−/−) mice in two steps, generating progeny including FXRβ−/− and aP2-hFXR mice on a FXRβ−/− background. Male mice used in this study were housed in a light(12:12)- and temperature (21°C)-controlled facility and received laboratory chow (RMH-B) and water ad libitum. When indicated, mice were fed a high-fat diet containing 60% calories from fat, composed of 36weight% beef fat (diets were obtained from Abdiets, Woerden, The Netherlands). Fat and lean mass was determined by MRI (Minispec, LF 90 II, Brüker, Billerica, USA). 4-hour fasted animals were terminated under anaesthesia, organs and tissues were carefully dissected and weighed. All experimental procedures were approved by the review board of the Animal Care and Use Committee of the Groningen University in accordance with local regulations for use of experimental animals.
**Histology and Adipocyte Size Determination**

The samples from liver and WAT fat depots were fixed in 4% neutral buffered paraformaldehyde, embedded in paraffin, cut into 4-μm sections, and stained with either hematoxylin/eosin or picrosirius red staining by standard procedures. Cell area of three times sixty adipocytes per animal (n = 6-8/group) was quantified using image analysis software (Qwin, Leica, Wetzlar, Germany). Crown-like structure abundance was determined in H&E-stained sections by counting CLS prevalence/mm² in whole sections of at least 10 mm².

**Indirect Calorimetry**

Mice were placed in an open-circuit indirect calorimeter system 24 hours prior to the start of the experiment with free access to water and food (TSE systems GmbH, Bad Homburg, Germany). Flow rates were measured and controlled with a mass flow controller. O₂ and CO₂ concentrations of dried inlet and outlet air from each chamber were measured every 10 minutes. Infrared light-beam frames surrounding the home cage measured activity in the x and y direction. Data were analyzed using LabMaster software (TSE systems GmbH, Bad Homburgh, Germany). Energy expenditure was calculated using the following equation: EE (kcal/hr) = (3.941*VO₂ (ml/hr) + 1.106*VCO₂ (ml/hr))/1000.

**Fat Absorption**

Fat absorption was determined as described previously (62).

**Hepatic de novo lipogenesis**

Labeling of the acetyl-CoA pool was assessed by providing [1-¹³C]-acetate (Isotec/Sigma-Aldrich, St. Louis, MO, USA) in drinking water as previously described by Jung et al. (63). Lipids in liver homogenates were fractionated using isolute NH₂ columns (Biotage AB, Uppsala, Sweden) and subsequently converted to their pentafluorobenzyl (PFB) derivatives.
GC-MS measurements of fatty acids and calculations of *de novo* lipogenesis and chain elongation of fatty acids were performed essentially as described (64). Pre-existing fatty acids (%) were calculated by the following equation: 100% - *de novo* synthesis (%) - chain elongation (%) originating fatty acids.

**Real Time Quantitative Reverse Transcription-PCR**

Total RNA was isolated from tissues using TRRireagent (Sigma-Aldrich, St. Louis, MO, USA). cDNA was synthesized by reverse transcription using reverse transcriptase and random primers according to the manufacturer’s protocol. mRNA expression levels were analyzed by means of real-time PCR on a 7900HT FAST real-time PCR system using FAST PCR master mix and MicroAmp FAST optical 96 well reaction plates (Applied Biosystems Europe, Nieuwerkerk a/d IJssel, The Netherlands) using Sybr green and ΔΔCT for all genes except *Fxr* that could only be detected using Taqman. PCR results were normalized to 36b4 (*Rplp0*) mRNA levels. Primers and probe sequences are listed in Supplemental Table S1.

**Liver and plasma parameters**

Hepatic lipids were extracted from homogenized livers in ice-cold PBS, lipids were extracted according to Bligh and Dyer (65). Lipid profiles in liver and plasma were determined using commercially available kits for free fatty acids, triglycerides and cholesterol (Wako, Neuss, Germany and Roche, Mannheim, Germany). Blood glucose and insulin concentrations were measured using a EuroFlash meter (Lifescan Benelux, Beerse, Belgium) and a ELISA (Mercodia, Uppsala, Sweden), respectively. For analysis of glucose kinetics a trace amount of labelled 13-C glucose was injected ip into 9-hour fasted mice and total glucose was measured every 10 minutes and blood spots were collected. Glucose kinetics were determined according to (40). Adipokine array was performed according to the manufacturer’s instructions (R&D systems, Minneapolis USA), using 50µl of pooled plasma from 6 animals per group.
Plasma bile acids

Bile acid concentration in plasma was measured in 25 μL of homogenized plasma. An internal standard containing D4-cholate, D4-chenodeoxycholate, D4-glycocholate, D4 taurocholate, D4-glycochenodeoxycholate and D4-taurochenodeoxycholate was added. Samples were mixed and centrifuged at 15900g. The supernatant was evaporated under vacuum at 40°C and reconstituted in 100 μl of 50% methanol. Bile acid profile was measured using liquid chromatography tandem MS (LC-MS/MS) as described (66).

Affymetrix microarray analysis

For the microarray analysis, total RNA was prepared from white epididymal (WAT) adipose tissue of 10-weeks old wild-type and aP2-hFXR-TG mice (n = 4/group) using TRIreagent (Sigma-Aldrich, St. Louis, MO, USA). RNA was further purified using RNeasy MinElute micro columns (Qiagen, Venlo, the Netherlands). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) using 6000 Nano Chips according to the manufacturer’s instructions. Five hundred nanograms of RNA were used for one cycle cRNA synthesis (Affymetrix, Santa Clara, CA). Hybridization, washing and scanning of Affymetrix Gene chip Mouse Gene 1.0 ST arrays was done according to standard Affymetrix protocols. Quality control, normalization (VSN), prefiltering (fold change 1.1) and statistics (IBMT) were performed in MADMAX (67) A list of significant changed annotated genes, including FDR-corrected p-values (5%) was generated. All microarray data reported are described in accordance with MIAME guidelines and are available in the GEO database (GSE37248) Identification of overrepresented functional categories among responsive genes and their grouping into functionally related clusters was performed using DAVID 6.7 Functional Annotation Clustering tool (68) and Gene Set Enrichment Analysis (69). Transcription factor analysis was performed with Metacore, Clarivate Analytics.
Adipose tissue culture and CILAIR

At 14-weeks of age, wild type (n = 6) and aP2-hFXR (n = 5) mice were sacrificed. Epididymal WAT was flushed with saline solution to remove blood from the tissue and carefully dissected. The adipose tissue culture protocol is described (70). The CILAIR protocol was described previously (32). Briefly, after mincing (20-80 mg pieces), washing, filtering and centrifuging the adipose tissue, the tissue was weighed and tissue pieces of individual wild-type and aP2-hFXR-TG mice were pooled and divided over three wild-type dishes (1.2 gram/dish in 12 ml medium) and three aP2-hFXR-TG dishes (0.75 gram/dish in 7.5 ml medium). Dishes with adipose tissue were incubated at 37°C at 5% CO₂ with lysine and arginine-free DMEM medium (D944; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 50 μg/ml gentamicin (15750; Invitrogen, Breda, The Netherlands), 100mg/l Leucine-HCl (L8912; Sigma-Aldrich, St. Louis, MO, USA) and 200 mg/l Proline (P5607; Sigma-Aldrich, St. Louis, MO, USA) to deplete the ¹³C lysine and arginine pool in the tissue. The medium was renewed after 1, 16, 20 and 23.5 hrs. After the last wash, all six dishes received fresh DMEM medium containing 100 mg/l L-[¹³C₆, ¹⁴N₂]lysine and 100 mg/l [¹³C₆, ¹⁵N₄] arginine (Cambridge Isotope Laboratories, Andover, CT, USA). Tissues were maintained in culture for an additional 24 hours to allow incorporation of the label into newly synthesized proteins. Thereafter media were collected and stored at -80°C until analysis. After concentration of the culture media to a final volume of approximately 30 μl by ultrafiltration, medium samples were fractionated by SDS-PAGE on 4-12% bis-Tris gel with a MOPS buffer (NuPAGE®-Novex, Invitrogen, Breda, The Netherlands). Bands were visualized and whole lanes were excised into 25 pieces that were processed for tryptic digestion. Each gel piece was washed, in-gel reduced, washed and rehydrated for 20 min by adding 5 ng/µl of modified trypsin (Promega, Madison, WI, USA) in 20 mM ammonium bicarbonate. Digestion was carried out overnight at 37 °C. After digestion the peptides were extracted from the gel by drying the gel pieces by speed-vac, rehydrating with 0.1% TFA (trifluoroacetic acid) and dehydrating with 0.1% TFA in 100% ACN. The extracted peptides were dried by speed-vac and re-dissolved in a MS compatible solvent (0.1% formic acid). Separation of the resulting tryptic peptide mixtures was performed by nanoscale reversed-phase LC-MS/MS. Protein identification and data analyses were performed as described (32).
Minor adjustments made to this protocol are:

- Selected mass range of 300–1100 m/z in Analyst QS 1.1 software (Applied Biosystems, Carlsbad, CA, USA).
- Selected three abundant charged peptides were dynamically excluded for 90 sec with 50 mmu mass tolerance
- ProteinPilot 3.0 software (Applied Biosystems, Carlsbad, CA, USA) using the UniprotKB/Swiss-Prot database was used to generate peak lists. Options that were chosen within the program were: label, Lys+6 and Arg+10; Cys alkylation with iodoacetamide; digestion with trypsin; gel-based identification; species, *Mus musculus*; identification focus for biological modifications; thorough search.

**Western blot**

Tissue lysates were prepared in RIPA lysis buffer (Tris-HCL pH=8, NaCl 138 mM, NP40 1%, KCl 2.7 mM, MgCl2 1 mM, Glycerol 5%, EDTA 5 mM, Na3VO4 1 mM, NaF 20 mM, DTT 1mM and protease inhibitor) and protein concentrations were quantified using the RC DC assay (Bio-Rad, Hercules, USA). Protein samples were subjected to SDS-PAGE (15% gels) and transferred to nitrocellulose using Trans-Blot® TurboTM transfer system (Bio-Rad). Membranes were blocked for 1 hour at room temperature) in PBS containing 0.1% Tween and 2% milk powder and incubated for 16 hours with primary antibodies at 4°C. Antibodies used in this study are: anti-Lamin A/C (Cell signaling, USA), anti-Col-1 (Southern Biotech, Birmingham, USA), and anti-GAPDH (Calbiochem, San Diego, USA). Proteins were detected by incubating the blot with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (Life science, NA934) or HRP-conjugated rabbit anti-mouse (Dako, p0260) IgG for 1 hour at room temperature. Bands were visualized using Supersignal West Dura substrate (Thermo Scientific, Waltham, USA) and ChemiDoc (Bio-Rad). Image Lab software (Bio-Rad) was used for densitometry.
**Statistical analysis**

Data are presented as mean +/- SEM. Differences between groups were tested by non-parametric Mann-Whitney U-test using Graphpad Prism 5.00 software package (GraphPad Software, San Diego, CA, USA). All values are given as means ± sem. Significance was indicated as *P < 0.05, **P < 0.01, ***P < 0.001.
Results

*aP2-hFXR mice have reduced body weight and enlarged adipocytes*

To directly explore the role of FXR in adipose tissue, we generated transgenic mice that overexpress FXR (NR1H4) under control of the aP2 (Fabp4) promoter. Specifically, a transgenic construct containing the mouse aP2 promoter region and the human FXR coding sequence was microinjected into fertilized mouse eggs (Supplemental Figure S1A). Transgenic founders were produced and characterization of two independent transgenic lines, expressing the highest levels of total FXR mRNA in fat tissues, revealed highly similar phenotypes. In the transgenic line used for these experiments (designated aP2-hFXR), total FXR mRNA expression level was increased 5.8, 3.1 and 3.2 fold in epididymal, retroperitoneal and subcutaneous inguinal white adipose tissue (eWAT, rWAT and iWAT), respectively, but expression was still modest when compared to expression of endogenous FXR in liver and ileum (Figure 1A).

Body weights of 14-weeks-old male transgenic mice were ~20-30 percent lower than those of control mice (Figure 1B). Food intake was slightly reduced in aP2-hFXR mice (Figure 1C). The decrease in body weight is unlikely due to reduced intestinal absorption efficiency, as dietary fat absorption was unaffected (Supplemental Figure S1B). A small but significant difference between aP2-hFXR mice and controls was observed in locomotor activity during the light fase (Figure 1D). As effects of absence of FXR in whole-body FXR<sup>−/−</sup> mice on carbohydrate metabolism were most apparent during fasting and refeeding (28,29), indirect calorimetry was performed during fed, fasted and re-fed conditions. Energy expenditure did not significantly differ between both groups under these conditions, also not during fasting and re-fed conditions (Figure 1E, Supplemental Figure S1C). Growth curves showed that the body weight difference was already apparent from weaning onwards (Supplemental Figure S1D). Surprisingly, the reduced weight of aP2-hFXR mice compared to controls was not only due to a reduction in fat mass; reduced lean body mass accounted for most of the difference (Figure 1F). WAT depot weights of chow-fed aP2-hFXR mice were comparable to those in wild type controls (Figure 1G), particularly when normalized to body weight (data not shown). Histological examination of adipose tissues however, revealed that adipocytes from chow-fed aP2-
hFXR animals were markedly hypertrophic (Figure 1H for eWAT, Supplemental Figure S1E for rWAT and iWAT).

To determine whether adipose tissue appearance is already affected early in development, WAT depots of very young aP2-hFXR mice were examined. Adipose depot development initiates just prior to birth and continues in the weeks thereafter, depending on the depot, and therefore WAT of very young mice provides the opportunity to focus on adipose tissue that predominantly develops by adipogenesis (30). rWAT and eWAT weights of aP2-hFXR mice were decreased compared to wild type controls already at 3 weeks of age (Figure 1I), despite a comparable histological appearance (Supplemental Figure S1F). Taken together, these results indicate a role for FXR in early life WAT development and functioning.

As aP2-driven expression has been reported to also affect macrophages, depending on the size of the promotor in the construct and the integration site (31), we isolated intraperitoneal macrophages from aP2-hFXR and wild type mice. Despite the fact that human FXR expression could indeed be detected, expression of inflammatory and FXR markers was not affected in these cells (Figure S1G). Expression levels of monocyte attractant protein 1 (Mcp1), and cluster of differentiation 68 (Cd68), a macrophage marker, were similar in eWAT of aP2-hFXR mice and controls (Figure S1H), indicating no major effects of aP2-hFXR expression on macrophage attraction. To determine whether hFXR expression alters endocrine functions of adipose tissues, adipokine levels were analyzed in pooled plasma samples. We observed a 10-50 percent reduction in abundance for the majority of proteins that could be detected, including several adipokines that have been associated with inflammation and insulin resistance, such as dipeptidyl peptidase 4, soluble ICAM-1, macrophage colony-stimulating factor, pentraxin-2 and resistin (Figure S1I). Also the classical adipokines leptin and adiponectin showed decreased levels, whereas fibroblast growth factor 21, that positively affects several aspects of metabolic syndrome was one of few proteins with increased abundance (+34%).

Since bile acids are the natural ligands for FXR, we analysed plasma bile acid levels and, unexpectedly, found elevated plasma bile acids in aP2-hFXR mice with altered composition. Particularly with substantially higher levels of cholate and taurocholate in the transgenic mice compared to controls were found (Figure 1J).
Hepatic expression levels of the major bile acid synthesis genes were analysed, showing that *Cyp7a1* and *Cyp8b1* expression levels in aP2-hFXR mice were similar to those in control animals and in line, no increased expression of *Shp* (*Nr0b2, small heterodimer partner*) was observed (Supplemental Figure S1J). Minor but significant decreases were observed for *Cyp27a1* (-18%) and *Cyp2c70* (-23%) mRNA levels. A more pronounced, 2-fold decrease was observed in expression level of the hepatic bile acid uptake transporters *Ntcp* and *Oatp1a1* (Figure S1J). Plasma aspartate transaminase (AST) levels were unaffected in aP2-hFXR mice while alanine transaminase (ALT) showed a slightly elevated trend but remained very close to baseline (5U/ml for controls and 14U/ml for aP2-hFXR, data not shown).

**Adipose FXR stimulates extracellular matrix remodeling**

To further establish the physiological role of FXR in WAT, adipocyte size distribution was analysed in the different depots. A clear shift in adipocyte size distribution as well as average adipocyte size was observed in chow-fed aP2-hFXR mice compared to controls (Figure 2A eWAT, Supplemental Figure S2A rWAT and iWAT). As the reduced adipocyte size in FXR<sup>-/-</sup> mice was one of the drivers of our initial interest towards investigating adipose FXR (24), aP2-hFXR mice were crossbred to whole body FXR knockout mice. These mice showed similar aspects in their phenotype as hFXR adipose overexpressors on the wild type background did, including ~20-30 percent lower body weights, stemming from reduced lean mass (Supplemental Figure S2B) and comparable locomotor activity and energy expenditure as FXR<sup>-/-</sup> controls (Supplemental Figure S2C,D). Adipose depot weights of FXR<sup>-/-</sup> aP2-hFXR mice were similar to controls (Figure 2B), yet, adipocyte hypertrophy was again apparent (Figure 2C, Supplemental Figure S2E). This hypertrophy was, however, somewhat less pronounced when compared to the hFXR-induced phenotype in the wild type background (Figure 2D, Supplemental Figure S2E,F).
To gain insight in the mode(s) of action by which the transcription factor FXR acts in WAT, Affymetrix microarray analysis was performed on eWAT samples obtained from chow-fed aP2-hFXR and wild type mice. A total of 1372 genes were found to be differentially expressed. Annotation analysis using DAVID and Gene Set Enrichment Analysis (GSEA) showed that among the most significantly affected cellular components and enriched gene sets, the extracellular matrix (ECM) seemed to be clearly affected by the presence of aP2-hFXR (Figure 3A). Several collagen-encoding genes were upregulated, including the collagen VI (but not the endotrophin-encoding a3 type), collagen I, IV and XV genes, as well as other ECM factors like elastin, biglycan and lumican, and ECM-modifying genes, such as cathepsins and metalloproteinases (Supplemental Table S1).

Altered expression or activity of the master regulators of adipogenesis, peroxisome proliferator-activated receptor (PPARγ) and CAAT/enhancer-binding protein (C/EBPα) could in theory account for the inability of adipose depot expansion in aP2-hFXR mice. A 26% and 33% reduction in expression of these genes was observed. However, no striking differential expression of PPARγ target genes between the groups were observed either (Supplemental Table S2). Wnt/β-Catenin signalling has been shown to be increased in FXR−/− mice. In line with the reported downregulation of secreted inhibitors of Wnt signalling Sfrp1 and Sfrp5 in FXR−/− mice (26), we observed upregulation (2.01-fold and 2.65-fold, respectively) of these genes in eWAT of aP2-hFXR mice. However, downregulation of Wnt signaling target genes as observed in vitro in preadipocytes (26) was not evident (Supplemental Table S2).

In line with the unaltered expression levels of Mcp1 and Cd68 (see Supplemental Figure S1H), eWAT transcriptomics showed no differential expression of established inflammation markers.

To assess the role of FXR in ECM remodeling in more detail, quantitative profiling of the epididymal WAT secretome was performed ex vivo, resulting predominantly in identification of increased protein levels or proteins only identified in WAT from aP2-hFXR mice and a limited number of proteins with decreased levels (Figure 3B, Supplemental Tables S3, S4)(32).
Overrepresentation of specific collagen families such as fibril-forming collagens (COL1A1, COL5A2), fibril-associated collagens (FACIT) (COL14A1, COL15A1) and beaded filament-forming collagens (COL6A1, COL6A5) and ECM components like laminin, fibronectin, biglycans, dermatopontin and cadherin-5 were observed in aP2-hFXR WAT secretomes, in line with the outcome of transcriptomics analysis.

To determine whether the observed increase in ECM protein synthesis actually leads to fibrosis in vivo and is not compensated by ECM degradation, specific fibrosis staining was performed of eWAT and iWAT harvested from both chow-fed wild type and FXR⁻/⁻ mice expressing aP2-hFXR and compared with the respective controls. Disorganized ECM was apparent in iWAT from the transgenic mice with loss of characteristic septa that subdivide the tissue into subsections (Figure 4A, Supplemental Figure S3)(33). In eWAT, accumulation of collagen was observed that predominantly surrounded individual adipocytes (i.e., pericellular fibrosis, Figure 4B, Supplemental Figure S3). COL1A1 protein level was also elevated in eWAT of FXR⁻/⁻ aP2-hFXR mice and, in line with the secretome analysis (see Figure 3), COL1A2 was not (Figure 4C,D). Lamin-A, a protein that scales with tissue stiffness (34), was increased as well (Figure 4C,D).

FXR expression in adipose tissue leads to redistribution of body fat during high-fat diet feeding

Next we examined the effects of FXR overexpression on WAT functioning in mice with a positive energy balance induced by feeding a high-fat diet for 6 weeks (HFD, 60% of calories from fat). Whereas WAT depots of control mice expanded (~2.5 fold, Figure 5A), those of aP2-hFXR mice remained similar in size to chow-fed conditions (only 1.1- to 1.4- fold expansion, compare dotted line to bar). Adipocytes from HFD-fed wild type mice were markedly larger than those from mice fed on chow, while adipocytes from aP2-hFXR mice remained as hypertrophic as observed during chow-fed conditions (Figure 5B,C, Supplemental Figure S4A). Both absorption of dietary fat and locomotor activity were unaffected and, in contrast to chow-fed conditions, cumulative food intake was similar in both groups (Supplemental Figure S4B-D). No differences in energy expenditure were observed between the groups either (Supplemental Figure S4E).
This unexpected finding on inability for WAT to expand, was subsequently also observed in an ageing experiment. Mass of WAT compartments was significantly increased by ~2.5 fold in aged (1 year old) chow-fed wild type mice when compared to 14-weeks old mice. In contrast, ageing of aP2-hFXR mice hardly induced any expansion of WAT depots, that, like the aged controls contained hypertrophic adipocytes with even smaller size compared to young aP2-hFXR mice. (Figure 5D-F, Supplemental Figure S4F).

FXR−/− mice were shown to be resistant to diet-induced obesity (27) and FXR−/− aP2-hFXR mice were therefore also fed the HFD. This resulted in similar weight gains (5.0g or 19%, vs 5.3g or 23% for FXR−/− and FXR−/− aP2-hFXR respectively, Figure 5G), indicating that adipocytic FXR is not responsible for the suppressive effect of FXR ablation on development of diet-induced obesity. aP2-driven FXR overexpression in the FXR−/− background did result in a small but significant increase in adipocyte size in eWAT and iWAT but not in rWAT after 6 weeks HFD (Figure 5H,I, Supplemental Figure S4G). Adipose depot size, however, remained similar to chow-fed conditions and lacked the expansion observed in FXR−/− controls (Figure 5G). Fibrosis staining again showed a disorganized ECM in iWAT of FXR−/− aP2-hFXR mice, however, as expected, septa were also not readily observed in FXR−/− controls after HFD (Figure 5J). Similar to chow-fed mice, fibrosis staining was more pronounced in eWAT of FXR−/− aP2-hFXR mice, however, also in FXR−/− control animals fibrotic patches became apparent after high-fat diet feeding, as occurs in wild type animals as well (Figure 5J,K)(33). In line, the increased expression of ECM-encoding genes observed in eWAT of chow-fed aP2-hFXR mice was not apparent anymore after HFD in eWAT of FXR−/− aP2-hFXR compared to FXR−/− controls with only an increased trend for Col1a1, indicating that the development of fibrosis occurred already early during development on standard chow conditions and is matched by the control animals during a positive energy balance (Supplemental Figure S4H).

Similar to the unaltered expression of inflammatory markers in eWAT and macrophages on the wild type background, expression of Cd68, Tnfa, Mcp1 and Il10 in eWAT and liver of chow- or HFD-fed FXR−/− aP2-hFXR mice was not altered compared to controls. Crown-like structure abundance also remained unaffected (Supplemental Table S5).
Adipocytic FXR-induced ectopic lipid accumulation aggravates insulin resistance

To determine whether the impaired expansion capacity of adipose depots in aP2-hFXR mice has adverse metabolic consequences, lipid contents in plasma and organs were analysed. Plasma free fatty acids tended to be elevated in chow-fed FXR⁻/⁻ aP2-hFXR mice when compared to FXR⁻/⁻ controls and were significantly elevated after HFD (Figure 6A). Free fatty acids were also elevated in aP2-hFXR mice on wild type background relative to controls both on chow and on HFD (Supplemental Figure S5A). After HFD feeding for 6 weeks, ectopic lipid accumulation in both liver and muscle tended to occur in hFXR overexpressing mice on wild type background as well, suggesting that suppression of adipose depot expansion in the presence of adipocytic FXR leads to fat accumulation, predominantly in the liver (Supplemental Figure S5B-D). Of note, the livers of chow-fed aP2-hFXR mice appeared very similar to those of control animals, confirming the absence of aminotransferases in plasma and liver damage. To exclude indirect effects on hepatic lipid accumulation, e.g., due to dysregulation of hepatic acetyl-CoA metabolism leading to excess hepatic lipogenesis (35), the origin of hepatic fatty acids was determined. Quantification of de novo lipogenesis in vivo by a mass isotopomer distribution analysis (MIDA) approach (36), showed virtually identical amounts of fatty acids derived from de novo lipogenesis and from chain elongation, indicating that stimulation of these pathways does not drive the accumulation of hepatic triglycerides in aP2-hFXR mice on HFD (Supplemental Figure S5E). This observation, combined with elevated free fatty acid levels in plasma, strongly suggests that hepatic fat accumulation is due to overflow of adipocytic fat stores.

A similar trend in hepatic triglyceride accumulation after 6 weeks HFD was observed in FXR⁻/⁻ aP2-hFXR mice compared to FXR⁻/⁻ controls (Supplemental Figure S5F,G). To elaborate on these metabolic consequences, we next extended the duration of a positive energy balance to 3 months in FXR⁻/⁻ and FXR⁻/⁻ aP2-hFXR mice, since FXR⁻/⁻ mice have been reported to be resistant against diet-induced obesity (27). To test whether these mice simply store less fat, total fat mass was determined by MRI, which showed that actually more fat accumulated in FXR⁻/⁻ aP2-hFXR mice than in FXR⁻/⁻ controls after this prolonged period of HFD feeding (Figure 6B). Within this period, significantly more
triglycerides and cholesterol accumulated in livers of FXR\textsuperscript{+/ -} aP2-hFXR mice than in those of FXR\textsuperscript{+/ -} mice (Figure 6C-E).

Plasma cholesterol levels were slightly elevated in FXR\textsuperscript{+/ -} aP2-hFXR mice, whereas plasma triglycerides remained similar to FXR\textsuperscript{+/ -} controls (Figure 6F,G). Muscle triglyceride content was significantly elevated in FXR\textsuperscript{+/ -} aP2-hFXR mice after 3 months on HFD (Figure 6H). The FXR\textsuperscript{+/ -} aP2-hFXR mice tended towards elevated plasma bile acids after 3 months on HFD, most probably due to the known dominant effect of hepatic FXR ablation, that shifts the bile acid composition towards predominantly taurocholate in both groups (Figure 6I, Supplemental Figure S5I)(37,38).

The difference in adipose depot size between both groups remained similar to the difference observed after 6 weeks HFD feeding, indicating that the storage capacity of WAT depots had rapidly become limiting in the FXR\textsuperscript{+/ -} aP2-hFXR mice, whereas in the FXR\textsuperscript{+/ -} control animals the positive energy balance could be matched by adipose storage capacity during the experiment (Supplemental Figure S5J).

Ectopic lipid accumulation, accompanied by increased concentrations of intermediates of lipid metabolism such as diacylglycerols and ceramides, is one of the drivers of insulin resistance (39). Therefore, we analysed glucose kinetics by a dynamic stable isotope test. Compared to FXR\textsuperscript{+/ -} controls, HFD-fed FXR\textsuperscript{+/ -} aP2-hFXR mice showed similar blood glucose levels, yet, insulin levels were 3-fold higher (Figure 6J,K). Of note, after 6 weeks of HFD insulin levels were unaltered in FXR\textsuperscript{+/ -} aP2-hFXR mice compared to controls (also on the wild type background Supplemental Figure S5K,L), indicating that the lack of adipose expansion precedes elevated insulin levels after prolonged obesogenic conditions.

Determination of glucose kinetics under normoglycemic conditions, using a stable isotopically-labelled glucose tracer (40), showed that metabolic clearance rates and glucose production rates were similar in aP2-hFXR FXR\textsuperscript{+/ -} and FXR\textsuperscript{+/ -} control mice, yet, at the expense of 3-fold increased insulin levels due to a marked hepatic and peripheral insulin resistance (Figure 6L-N). Overall, these data indicate that moderate aP2-controlled overexpression of human FXR in fat tissues results in adipocyte hypertrophy and limited white adipose tissue expansion capacity when challenged with high-fat diet, leading to ectopic lipid accumulation and insulin resistance.
Discussion

These studies establish that the bile acid-activated nuclear receptor FXR is an important determinant of fat tissue architecture and function. We show that transgenic mice moderately overexpressing FXR under the control of the aP2 (Fabp4) promoter during standard (chow) conditions have WAT with enlarged adipocytes. On HFD, as well as at moderately old age, these mice show very limited expansion of WAT depots and an inability to further increase adipocyte size. In accordance with a limited storage capacity of WAT due to localized FXR overexpression, ectopic lipid deposition occurred during a positive energy balance, on both the wild type and FXR−/− backgrounds. Extensive extracellular matrix (ECM) remodeling may be involved in this unexpected action of FXR in regulating adipose architecture.

Although temporal dynamics of specific WAT depot expansion differs and different developmental stages have distinct molecular underpinnings of adipogenesis (30,41,42), FXR stimulated hypertrophy and limited expansion in all depots analyzed, and at different ages, indicating a fundamental role of this nuclear receptor in adipose architecture.

In line with the “critical fat cell size” hypothesis (43), the hypertrophic adipocytes in aP2-hFXR mice did not increase further in size during a positive energy balance and depot size also did not increase: therefore, hyperplasia must be suppressed under these conditions. This was confirmed by the reduced depot size of FXR-overexpressing mice shortly after birth, although FXR has been proposed to be a positive regulator of adipogenesis in vitro (25,26). The adipose micro-environment in the in vivo situation therefore seems to predominate over potential positive effects of FXR activity on adipogenesis, however, to confirm that FXR does not affect Wnt signaling in vivo requires a more thorough investigation of preadipocytes in WAT. Interestingly, prolonged treatment with an FXR agonist during obesogenic conditions also seems to result in adipocyte hypertrophy compared to control diet but also compared to high-fat diet (44).

The observation that in aged mice overexpressing FXR, expansion of adipose depots was also limited and that adipocyte size in eWAT was even decreased compared to young mice (compare Figure 2A to 5F) is in line with inhibition of adipose plasticity by FXR. The eWAT depot is known to have a high dependency on newly formed cells, a process that declines with age (45,46).
FXR expression was reported to decrease in WAT during genetic and diet-induced obesity in mice (24). We now provide evidence that this effect may in fact liberate WAT plasticity, providing increased storage capacity upon excessive energy intake.

The wild type and FXR⁻/⁻ backgrounds did result in subtle differences concerning the effects on WAT expansion and adipocyte size by FXR overexpression (i.e., compare Figure 5C to 5I), suggesting that external FXR-mediated cues on adipose FXR function exist. Of note, although the reported reduced adipocyte size in FXR⁻/⁻ mice was one of the major reasons for us to focus on adipocytic FXR (24), the complete FXR knockout model used in this study displayed similar adipocyte size as wild type mice did (compare Figure 2A to 2D)(38) and thus lacks the smaller adipocytes observed earlier in FXR⁻/⁻ mice generated by Cre-mediated deletion of the last exon (24). Possibly, the latter model might still produce a truncated form of FXR that contains the DNA-binding domain (38). When comparing the wild type and FXR-deficient backgrounds, however, the large shift in bile acid pool size and composition due to lack of hepatic and intestinal FXR could affect the function of adipose FXR, as ligand availability in plasma is dramatically altered (47). As observed previously (48), we also identified expression of Organic Anion Transporting Polypeptide 2B1 (OATP2B1, SLCO2B1), a transporter with ubiquitous expression that may facilitate adipocytic bile acid uptake. Unexpectedly, adipose FXR overexpression resulted in elevated plasma bile acids with a more hydrophobic composition, which may generate a feedforward loop towards adipose FXR activation. Analysis of expression of hepatic bile acid synthesis genes on which both intestinal and hepatic FXR activity converges, revealed only a minor reduction in Cyp7b1, Cyp27a1 and Cyp2c70 and no effect on Shp expression, or the major bile acid synthesis genes Cyp7a1 and Cyp8b1 that are regulated by SHP downstream of hepatic FXR. Yet, Nicp and Oat1a1 were over 2-fold downregulated, suggesting that impaired hepatic uptake of bile acids is responsible for the observed accumulation of bile acids in plasma. The underlying mechanism hereof still remains elusive and awaits further studies. Adipose tissue formation during late fetal and early postnatal life in humans is highly sensitive to the nutritional environment and coincides with the so-called ‘physiological cholestasis’ period in infants (elevated plasma concentrations of primary bile acids)(49).
Bile acids acting as endogenous ligand for FXR in adipose tissue that contribute to regulation of adipogenesis are therefore not inconceivable during this stage of development.

Adipose transcriptomics and \textit{ex vivo} secretome analysis revealed broad changes in the adipose extracellular matrix (ECM) upon FXR overexpression. Recent interest in the cues that determine the fate of stem cells towards a specific lineage have underlined the importance of the microenvironment in which these cells reside and in particular the properties of the ECM (50). The stiffness of the matrix defines the plasticity of the stem cell/pre-adipocyte population: nuclear Lamin A, a component of one of the mechano-sensing mechanisms, strongly scales with tissue stiffness (34).

Mastering these parameters of the microenvironment in which stem cells are directed towards a desired fate has been instrumental in enabling stem cell research, tissue regeneration and the production of organoids (51). Also in adipose tissue, ECM homeostasis and remodeling according to prevailing tissue needs is an important high-maintenance process, which is particularly evident in mature adipocytes (52).

Based on our results, we propose that FXR-induced alterations in ECM homeostasis dictate adipose hyperplasia and depot expansion. Control of the adipose microenvironment has recently also been shown to be regulated by a specific subset of adipocyte progenitors (53). These cells, that are either positive for platelet-derived growth factor receptor $\alpha$ or $\beta$, seem to derive from a common progenitor as the preadipocytes destined to mature into adipocytes. These cells therefore seem to be at the crossroad between healthy and unhealthy adipose tissue development (54,55). Despite the fact that the adiponectin promotor has become the preferred tool for generating adipocyte-specific knockout mice, the aP2-driven model applied in this study has the advantage that it also targets the adipocyte precursors. Whether FXR already alters the developmental trajectory of these progenitors with increased tissue stiffness as a consequence or acts a bit further down the line, for instance by altering this Lamin A-based sensing mechanism (56), remains to be answered.

Paradoxically, the Scherer laboratory revealed that, next to its infamous role in metabolic disease, inflammation of adipose tissue is actually required for healthy expansion as well (57).
Although the level of expression in adipose tissue is more than 1000-fold higher than in adipose-resident macrophages (58), we were able to detect human FXR mRNA in isolated macrophages in our model. However, no effect was observed on inflammatory markers or the abundance of crown-like structures, or even on the expression of FXR targets in macrophages, strongly suggesting that the off-target expression is very limited, although we cannot definitely rule out a role for macrophage FXR. One phenomenon in our model that could possibly be explained by non-adipocyte expression, is the highly intriguing reduction in lean body mass. Yet, we think that it is tempting to speculate that an adipocyte-derived factor is causal to this phenotype, we were unable to identify major changes in known adipokines that regulate lean mass development. Further studies are therefore required to determine its cause.

The increased plasma free fatty acid levels that were observed already under basal conditions suggests that WAT storage capacity is limiting but can systemically still be compensated for. Yet, exposure to a positive energy balance provokes ectopic lipid accumulation in liver and muscle. As adipose-derived fatty acid flux contributes 60-80% of the hepatic lipid influx in both mice and men, subtle changes in adipose output may lead to severe hepatic consequences over time (59,60). Recently, for instance, adipose tissue lipolysis rates were found to drive hepatic glucose production (35). The observed elevated plasma levels of free fatty acids in our model, indicative for an increased rate of lipolysis, did however not lead to increased glucose production. Yet, ectopic lipid accumulation was associated with evident insulin resistance, which delineates a pathophysiological relevance for adipocytic FXR in whole-body energy metabolism.

In conclusion, our studies established that the nuclear receptor FXR is a novel determinant of fat tissue architecture and function, among others by control of ECM remodeling, which adds to the extended list of FXR functions (2,5). We provide new insights into FXR-mediated maintenance of whole-body energy balance in addition to its well-described functions in liver and intestine which, in view of the current development of FXR agonists for treatment of human metabolic diseases, fat-specific actions might contribute to the actions of these compounds and warrants careful evaluation of their effects in adipose tissues.
Declaration of Interest

The authors have no competing interests to declare

Acknowledgements

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### Table 1 Primers used in the study

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<th>Forward</th>
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Downloaded from www.jlr.org at University of Groningen, on July 5, 2019
Figure 1 Adipose hFXR overexpressing mice are small and have hypertrophic adipocytes. (A) Quantitative real-time PCR of total FXR expression in white adipose tissue (WAT) depots (epididymal, retroperitoneal and inguinal), liver, ileum, kidney, adrenals, muscle and heart of wild type and adipocyte-specific FXR overexpressing mice (aP2-hFXR). (B) Body weight, (C) cumulative food intake from 6 to 13 weeks of age and (D) locomotor activity of wild type and aP2-hFXR mice. (E) Energy expenditure at 21°C measured over 12 hours light, 12 hours dark phase. (F) Lean and fat mass after 13 weeks. (G) WAT depot weights and (H) representative image of eWAT morphology showing adipocyte hypertrophy. (I) Depot size of rWAT and eWAT 3 weeks after birth of wild type and aP2-hFXR mice. (J) Plasma bile acid composition and total amount of wild type and aP2-hFXR mice. Bar represents 100 µm. All panels: N=6-8/group, data are represented as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.
Figure 2 Adipose hFXR increases adipocyte size without affecting adipose depot size (A)

Distribution of adipocyte size (in percentage) in eWAT and average adipocyte size in wild type and aP2-hFXR mice. (B) Body and WAT weight, (C) eWAT morphology and (D) Distribution and average of adipocyte size of FXR−/− and FXR+/− aP2-hFXR mice. All panels: N=6-8/group, data are represented as mean ± SEM, *p<0.05. Bar represents 100µm.
Figure 3 Adipose hFXR affects transcriptional and translational processes associated with the extracellular matrix formation. Functional annotation categorization of aP2-hFXR versus wild type eWAT transcriptomics, showing (A) David top 10 of enriched Cellular Components and Gene Set Enrichment Analysis. (B) Secretome analysis of eWAT ex vivo showing statistically significant decreased/increased protein levels as well as aP2-hFXR specific proteins identified according to cellular localization.
Figure 4 Adipose hFXR causes collagen deposition and alters adipose architecture. Collagen characterization in (A) iWAT and (B) eWAT of chow-fed FXR\(^{-/-}\) and FXR\(^{-/-}\) aP2-hFXR mice by Picosirius red staining. (C) Collagen I and Lamin-A/C protein levels and (D) quantification in eWAT of chow-fed FXR\(^{-/-}\) and FXR\(^{-/-}\) aP2-hFXR mice, N=6, data are represented as mean ± SEM. *p<0.05, **p<0.01. Bar represents 200 µm.
Figure 5 High-fat diet prevents further growth of hypertrophic adipocytes in aP2-hFXR mice and limits fat depot expansion. (A) Body and WAT weight of wild type and aP2-hFXR mice after 6 weeks high-fat diet. The dotted lines indicate WAT weights of chow-fed mice (presented in Figure 1) (B) eWAT morphology and (C) distribution and average of adipocyte size after 6 weeks HFD. Dotted lines indicate adipocyte size of chow-fed mice (D) Body and WAT weight of 1-year old chow-fed wild type and aP2-hFXR mice.
(E) eWAT morphology and (F) distribution and average of adipocyte size of 1-year old wild type and aP2-hFXR mice. (G) Body and WAT weight of FXR<sup>−/−</sup> aP2-hFXR mice after 6 weeks high-fat diet. Dotted lines indicate weights of chow-fed control animals (depicted in Figure 2) (H) eWAT morphology and (I) distribution of adipocyte size and average adipocyte size of FXR<sup>−/−</sup> aP2-hFXR mice after 6 weeks high-fat diet. Sirius red fibrosis staining of (J) iWAT and (K) eWAT after 13 weeks high-fat diet. N=6-9/group, data are represented as mean ± SEM, *p<0.05. Bar represents 100 µm.
Figure 6 Obesogenic conditions induces ectopic lipid accumulation and insulin resistance in aP2-hFXR mice. (A) Plasma free fatty acids levels of chow- and HFD-fed FXR\textsuperscript{+/−} and FXR\textsuperscript{−/−} aP2-hFXR mice. (B) Fat mass of FXR\textsuperscript{−/−} and FXR\textsuperscript{−/−} aP2-hFXR mice after 3 months HFD (C) Liver H&E staining showing lipid accumulation that is most apparent in the pericentral zone. Top scale bar (panel C) depicts 500µm, bottom scale bar in enlargement 200µm. (D) Hepatic triglycerides and (E) hepatic total cholesterol of FXR\textsuperscript{−/−} and FXR\textsuperscript{−/−} aP2-hFXR mice after prolonged HFD. (F) Plasma total cholesterol and (G) triglycerides of FXR\textsuperscript{−/−} and FXR\textsuperscript{−/−} aP2-hFXR mice after 3 months HFD. (H) Gastrocnemius triglycerides of FXR\textsuperscript{−/−} and FXR\textsuperscript{−/−} aP2-hFXR mice after 3 months HFD and (I) plasma bile acid composition. (J) 4-hour fasted plasma insulin and (K) blood glucose of chow- & HFD-fed FXR\textsuperscript{−/−} and FXR\textsuperscript{−/−} aP2-hFXR mice. (L) Metabolic clearance rate, (M) Turnover rate (that under these conditions equals glucose production) and (N) peripheral and hepatic insulin sensitivity during a whole-body glucose test of FXR\textsuperscript{−/−} and FXR\textsuperscript{−/−} aP2-hFXR mice after 3 months HFD. N=6-8/group, data are represented as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.