Hepatic glucocorticoid-induced transcriptional regulation is androgen-dependent after chronic but not acute glucocorticoid exposure

Buurstede, J.C.; Paul, S.N.; Bosscher, K. de; Meijer, O.C.; Kroon, J.

RESEARCH ARTICLE

THE FASEB JOURNAL
The Journal of the Federation of American Societies for Experimental Biology

Hepatic glucocorticoid-induced transcriptional regulation is androgen-dependent after chronic but not acute glucocorticoid exposure

Jacobus C. Buurstede[1] [ORCID] | Susana N. Paul[1] | Karolien De Bosscher[2] |
Onno C. Meijer[1] | Jan Kroon[1]

[1]Division of Endocrinology, Department of Medicine, Leiden University Medical Center, Leiden, The Netherlands

[2]Translational Nuclear Receptor Research, VIB Center for Medical Biotechnology, UGent Department of Biomolecular Medicine, Gent, Belgium

Correspondence
Jan Kroon and Jacobus C. Buurstede, Division of Endocrinology, Department of Medicine, Leiden University Medical Center, Albinusdreef 2, Leiden 2333 ZA, The Netherlands.
Email: j.kroon@lumc.nl and j.c.buurstede@lumc.nl

Funding information
JCB was funded with a PhD grant by the Board of Directors of Leiden University Medical Center

Abstract

Glucocorticoids exert their pleiotropic effects by activating the glucocorticoid receptor (GR), which is expressed throughout the body. GR-mediated transcription is regulated by a multitude of tissue- and cell type-specific mechanisms, including interactions with other transcription factors such as the androgen receptor (AR). We previously showed that the transcription of canonical glucocorticoid-responsive genes is dependent on active androgen signaling, but the extent of this glucocorticoid-androgen crosstalk warrants further investigation. In this study, we investigated the overall glucocorticoid-androgen crosstalk in the hepatic transcriptome. Male mice were exposed to GR agonist corticosterone and AR antagonist enzalutamide in order to determine the extent of androgen-dependency after acute and chronic exposure. We found that a substantial proportion of the hepatic transcriptome is androgen-dependent after chronic exposure, while after acute exposure the transcriptomic effects of glucocorticoids are largely androgen-independent. We propose that prolonged glucocorticoid exposure triggers a gradual upregulation of AR expression, instating a situation of androgen dependence which is likely not driven by direct AR-GR interactions. This indirect mode of glucocorticoid-androgen interaction is in accordance with the absence of enriched AR DNA-binding near AR-dependent corticosterone-regulated genes after chronic exposure. In conclusion, we demonstrate that glucocorticoid effects and their interaction with androgen signaling are dependent on the duration of exposure and believe that our findings contribute to a better understanding of hepatic glucocorticoid biology in health and disease.

KEYWORDS

androgen receptor, androgens, glucocorticoid receptor, glucocorticoids, liver

1 | INTRODUCTION

Glucocorticoids are steroid hormones that are produced in the adrenal cortex and secreted into circulation in a circadian manner or after a stressor. Glucocorticoids cause pleiotropic effects, ranging from modulation of memory consolidation and its well-known anti-inflammatory effects, to the regulation of glucose and lipid metabolism.[1–4] Glucocorticoids reach virtually all organs, most of which express considerable levels of the glucocorticoid receptor (GR).[5] The GR is a nuclear receptor which upon ligand binding translocates to the nucleus. Its main mode of action entails binding to specific motifs in the DNA called glucocorticoid response elements (GREs) to regulate expression of associated genes.[6] To exert its regulatory effects, the GR interacts with various coregulatory proteins to form a transcriptional complex, of which the composition influences the final outcome of transcription.[7] While GRs are globally activated after elevated glucocorticoid exposure, they exert specific effects dependent on tissue and cell type via complex regulatory mechanisms.[8,9]

In order for GR to exert tissue and cell type specific effects, its activity is regulated on multiple levels. Cellular availability of glucocorticoid ligands is regulated by transporter proteins in circulation and via enzymatic (in)activation by 11β-hydroxysteroid dehydrogenase type 1 and 2.[10,11] This provides a level of specificity by rendering certain tissues and cell types insensitive to glucocorticoids. GR signaling is also modulated by cell specific expression of co-activator and co-repressor proteins that are recruited into the GR transcriptional complex.[12] The effects of GR activation can be further tailored to the context by interactions with other transcription factors. This may include crosstalk between nuclear receptors, either through tethering, heterodimerization or even multimerization at the DNA.[13,14] However, crosstalk between nuclear receptors can also occur up- or downstream of DNA-binding (indirect crosstalk).[13]

Glucocorticoid effects can be sexually dimorphic, likely in interaction with sex hormones.[15–17] Crosstalk between the GR and known sex steroid receptors (androgen receptor [AR] and estrogen receptor alpha [ERα]) were described in cancer,[18,19] and such crosstalk might also underlie the diverging effects of GR activation in physiology as well as pathophysiology. In the liver, a pivotal target tissue of glucocorticoids, GR and AR are expressed at varying levels and these receptors are involved in the regulation of metabolic processes. Liver-specific GR knockout increases insulin sensitivity, while hepatic AR knockout decreases insulin sensitivity.[20,21] Sexual dimorphism also exists for liver diseases such as non-alcoholic fatty liver disease (NAFLD) which is more prevalent in men.[22] Glucocorticoid and androgen signaling both have been associated with the development of NAFLD and its progression towards hepatocellular carcinoma.[23,24] For glucocorticoid-induced transcription in the liver, we previously described a dependency on active androgen signaling for a select number of genes, but the extent of this phenomenon warrants further investigation.[25]

In the present study, we set out to determine the extent of glucocorticoid-androgen transcriptional crosstalk in the male mouse liver and how this crosstalk is influenced by the duration of exposure. We analysed the liver transcriptome after acute and chronic exposure to the GR agonist corticosterone (CORT) and the AR antagonist enzalutamide (ENZA). We reveal that the effects of chronic CORT exposure on the liver are partially androgen-dependent, while this dependency is not apparent after acute glucocorticoid exposure, indicating indirect glucocorticoid-androgen crosstalk. As a potential driving mechanism, we show that chronic glucocorticoid exposure upregulates hepatic AR expression via direct GR binding to the *Nr3c4* (*Ar*) gene.

2 | MATERIAL AND METHODS

2.1 | Animal experiments

All animal studies were approved by the ethical committee of Leiden University Medical Center.

2.2 | RNA-sequencing cohort after chronic corticosterone and enzalutamide exposure

Eight-week-old male C57B6/J mice were subcutaneously implanted with slow-release pellets containing 100 mg cholesterol (Vehicle; $N = 6$) or 20 mg corticosterone + 80 mg cholesterol (CORT; $N = 6$). An additional experimental group was implanted with pellets containing 20 mg corticosterone + 80 mg cholesterol and treated with 40 mg/kg/day enzalutamide via diet-supplementation (CORT_ENZA; $N = 5$). Implantation of CORT pellets was previously shown to result in continuous supraphysiological levels of CORT for the duration of the experiment[26] After 14 days, mice were sacrificed via CO_2 asphyxiation, perfused with ice-cold PBS for 5 minutes and livers were collected for RNA-sequencing analysis.

2.3 | RNA-sequencing cohort after acute corticosterone and enzalutamide exposure

Eight-week-old male C57B6/J mice were treated three times with solvent or with 40 mg/kg/day enzalutamide

with 12 h intervals. One hour after the last enzalutamide administration, mice were injected subcutaneously with 3 mg/kg corticosterone (in 5% EtOH, 95% PBS) or vehicle. Mice were sacrificed via CO_2 asphyxiation 3 h after corticosterone injection, perfused with ice-cold PBS for 5 min and livers were collected for RNA-sequencing analysis.

2.4 | Corticosterone slow-release pellets in adrenalectomized and intact mice

Eight-week-old male C57B6/J mice underwent bilateral adrenalectomy (ADX) to deplete endogenous glucocorticoids, and mice were placed on water containing 0.9% NaCl. During the surgical adrenalectomy procedure, slow-release pellets containing 100 mg cholesterol (ADX; $N = 8$), 3.75 mg corticosterone + 96.25 mg cholesterol (ADX + 3.75 mg CORT; $N = 8$) or 20 mg corticosterone + 80 mg cholesterol (ADX + 20 mg CORT; $N = 8$) were placed subcutaneously in the neck region. After 3 days, mice were sacrificed by CO_2 asphyxiation, blood was collected via a heart puncture, mice were perfused with ice-cold PBS for 5 min, and livers were collected for expression analysis of *Nr3c4* (*Ar*) by qPCR. We repeated this experiment in intact mice by implanting 100 mg cholesterol (Vehicle; $N = 6$) or 20 mg corticosterone + 80 mg cholesterol pellets (20 mg CORT; $N = 6$). After 4 days, mice were sacrificed by CO_2 asphyxiation, blood was collected via heart puncture, mice were perfused with ice-cold PBS for 5 min, and gonadal white adipose tissue, interscapular brown adipose tissue and quadriceps muscle were collected for *Nr3c4* (*Ar*) expression analysis. Livers were collected for qPCR, western blot and chromatin-immunoprecipitation-qPCR analysis.

In two separate cohorts, eight-week-old male C57B6/J mice were pre-treated with 60 mg/kg GR-specific antagonist Relacorilant (Corcept Therapeutics; $N = 5–6$),[27] 1 h prior to subcutaneous injection with 5 mg/kg GR-specific agonist dexamethasone phosphate (Merck); or mice were treated daily with 60 mg/kg Relacortilant (Corcept Therapeutics; $N = 6$) in combination with pellets containing 20 mg corticosterone + 80 mg cholesterol for 5 days. Mice were sacrificed by CO_2 asphyxiation, mice were perfused with ice-cold PBS for 5 min, and livers were collected for *Nr3c4* (*Ar*) expression analysis by qPCR.

2.5 | Time series cohort after acute corticosterone exposure

Eight-week-old male C57B6/J mice were injected subcutaneously with 3 mg/kg corticosterone (in 5% EtOH, 95% PBS) at 09:00. One group of naïve mice was

sacrificed without intervention for baseline determination. Corticosterone groups were sacrificed 3, 6 or 12 h after injection via CO_2 asphyxiation, blood was collected by heart puncture, mice were perfused with ice-cold PBS for 5 min and liver tissue was collected for *Nr3c4* (*Ar*) and *Nr3c1* (*Gr*) expression analysis by qPCR and western blot.

2.6 | Corticosterone measurement

Corticosterone levels were measured in plasma from heart puncture blood using a HS EIA kit (Immunodiagnostic Systems).

2.7 | Real-time quantitative PCR

Snap-frozen tissues were homogenized in Tripure (Roche) and total RNA was isolated according to the manufacturer's protocol. cDNA was synthesized from 1000 ng of RNA using random hexamers and M-MLV reverse transcriptase (Promega). Gene expression was assessed with RT-qPCR and normalized for housekeeping genes B2M and 36B4. Primer sequences are available upon request.

2.8 | Co-Immunoprecipitation

Co-Immunoprecipitation (co-IP) was performed on snap-frozen liver tissue from mice chronically exposed to corticosterone (20 mg CORT via slow-release pellets). Tissue was homogenized using a tissue homogenizer in NP-40 buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, and 1% NP-40 with protease/phosphatase inhibitor cocktail) and sonicated for 30" on/30" off for 6 cycles. Protein samples were pre-cleared with 50 µl Dynabeads (Invitrogen 10002D) and washed in NP-40 buffer. Per IP, 50 µl of BSA immobilized Dynabeads were incubated overnight with 5 µl anti-GR (Cell Signaling Technology D8H2) or 1 µl aspecific antibody (Flag M2; Sigma-Aldrich F3165) while rotating at 4°C. Afterwards, 150 µg of protein sample was added and incubated for another 2 h. Subsequently, IP samples and controls were lysed in sample buffer (4x Laemmli with beta mercaptoethanol) and denatured for 5 min at 95°C before analysis by western blot.

2.9 | Western blot

Protein expression of AR, GR, and UBE3A was detected by WES automated western blot (ProteinSimple), according to the manufacturer's instructions. In short, 0.8 mg/ml liver lysate or input and 4x diluted co-IP samples were loaded on

66–440 kDa separations modules (Biotechne) and incubated with the following primary antibodies: rabbit AR antibody (1:20 dilution; Santa Cruz Biotechnology sc-816), rabbit GR antibody (1:20 dilution: Cell Signaling Technology D8H2), rabbit UBE3A antibody (1:10 dilution: Abcam ab10488) and rabbit GAPDH antibody (1:20/50 dilution; Santa Cruz Biotechnology sc-25778); and secondary antibody: HRP anti-rabbit (1:100 dilution; ProteinSimple DM001).

2.10 | Chromatin immunoprecipitation-qPCR

Chromatin-immunoprecipitation (ChIP) was performed on 30 μm cryo-sections from snap-frozen liver tissue. Proteins were crosslinked head-over-head at room temperature with 2 mM DSG for 25 min and 1% formaldehyde for 20 min, and samples were quenched with 2 M glycine. Samples were washed and homogenized further using a tissue homogenizer. After resuspension in sonication buffer samples were sonicated 30″ on/30″ off for 6 cycles, 1% Triton was added and samples were washed. Input controls were set aside, 5 μl human control RNA and 5 μl human recombinant Histon2B was added to ChIP-samples, and these were aliquoted for IgG- and GR-ChIP. Protein A beads pre-incubated with IgG (5 μg/sample; Abcam ab37415) and GR (1 μg/sample; Cell Signaling 12041S) antibodies were added, and samples were rotated overnight at 4°C. The next day, beads were washed 10 times with RIPA buffer, and samples were eluted, reverse crosslinked and diluted in nuclease-free water. GR-enrichment at the *Nr3c4* (*Ar*) gene was determined with the following primer set: Fwd—CCCTTGTCCTTTGCCCTCTT; Rev—CAACCTCAGCCCAAGTCTGT.

2.11 | Liver lipid measurements

Lipids were extracted from liver tissue following a modified protocol of Bligh and Dyer.[28] In brief, approximately 50 mg of tissue was homogenized in 10 μl methanol per mg tissue and 1800 μl methanol: chloroform (1:3 v/v) was added to 45 μl of homogenate. Samples were vigorously vortexed and centrifuged (15 min; 20 000 g) to extract the lipids. The organic phase was dried twice under nitrogen, first re-dissolved in 100 μl 2% Triton X-100 in chloroform and finally in 100 μl water. Triglycerides, total cholesterol and phospholipid levels were determined using enzymatic kits (20767107322 and 03039773190 (Roche Diagnostics) and 3009 (Instruchemie), respectively). Protein concentrations were determined using the Pierce™ bicinchoninic acid assay kit (23225; ThermoFisher) and lipid levels were expressed as nmol/mg protein.

2.12 | RNA-sequencing

For RNA-sequencing (RNAseq) total RNA was isolated from liver tissue. For the chronic exposure cohort, the tissue was homogenized using a tissue homogenizer in lysis buffer of the NucleoSpin RNA kit (Macherey-Nagel). Total RNA was isolated according to the manufacturer's protocol and samples were send for transcriptome sequencing at BGI Genomics. For the acute exposure cohort, total RNA was isolated at BGI Genomics using the RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA quality of all samples was assess using the RNA 6000 Nano kit on a Bioanalyzer (Agilent) and all samples passed the quality criteria for sequencing (RNA Integrity Number >7.0 and 28/18s ratio >1.0). Stranded mRNA libraries were constructed and 100 bp paired-end sequencing was performed on the DNBseq platform resulting in >20 million reads per samples. RNAseq data has been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE180445.

2.13 | RNA-sequencing data analysis

The RNA-seq pipeline (version 4.1.0), published as part of BioWDL, was used for read quality control, alignment and quantification. BioWDL contains the main sequencing analysis pipelines and workflows developed at Leiden University Medical Center by the sequencing analysis support core with code being accessible at https://biowdl.github.io/.

Quality control was performed using FastQC and MultiQC. Reads were aligned to Mus Musculus genome version 10 (mm10) using STAR (version 2.7.3a). Tool settings used were: '--runThreadN' '4' '--outSAMunmapped' 'Within KeepPairs' '--twopassMode' 'Basic' with alignment between 90% and 95%. The gene-read quantification was performed using HTSeq-count (version 0.12.4). Tool settings used were: '--order' 'pos' '--stranded' 'reverse' resulting in 60%–75% of reads uniquely assigned to known genes based on Ensembl release 97 of mm10. HTSeq-count output files were merged into a count matrix per experiment as input for differential gene expression analysis.

DEseq2 (version 1.29.4) was used for normalization of the count data (median of ratio's method) and identification of differentially expressed genes.[29] For the differential expression analysis, all genes which were expressed in a minimum of four out of six replicates with >20 normalized counts for at least one of the groups were selected. Three samples were identified as outliers (sample 18 of the chronic cohort and samples L19A and L36A of the acute

cohort) and excluded from subsequent analysis. This resulted in 11 839 genes in the analysis for the chronic exposure experiment and 12 108 genes for the acute exposure experiment (Supporting dataset). Pair-wise comparisons of groups within experiments were analysed and a false discovery rate adjusted p-value of 0.05 was used as a cut-off for detection of differential gene expression. Principal component analysis was performed using DEseq2 and visualized with the pca3d package (version 0.10.2). Heatmaps of scaled, normalized counts were made with pheatmap (version 1.0.12). Gene ontology (GO) term enrichment analysis was performed with the ViSEAGO package (version 1.4.0), using fisher's exact test with 0.01 as a significance cut-off.

2.14 | Assessment of AR downstream signaling

A list of hepatic AR target genes identified by Zheng et al.[30] and a list of AR interacting proteins reported by Lempiainen et al.[31] were used to explore the effect of CORT on AR signaling. Transcriptome data of the corresponding genes after chronic CORT exposure was visualized as heatmaps.

2.15 | ChIP-sequencing analysis

Publicly available ChIP-sequencing data of GR at high endogenous CORT levels[32] and AR in unstimulated conditions[33] in the liver were utilized to identify genes potentially directly regulated by these transcription factors. All reported genomic loci of GR and AR (including promoter and enhancer regions) were annotated to the nearest transcription start site using homer[34] and corresponding genes were associated to the relevant transcription factor(s). Lists of GR and AR associated genes (irrespective of the genomic location of the corresponding binding site) were cross-referenced with subsets of differentially expressed genes and percentages of overlap were calculated.

2.16 | Single-cell expression data

Human liver single-cell RNA-seq data published by Aizarani et al. was utilized.[35] We extracted the data and visualized the main clusters of cell types and presence/absence of *NR3C1* (*GR*), *NR3C4* (*AR*) or both *NR3C1* (*GR*) and *NR3C4* (*AR*) expression per cell in t-SNE plots using the RaceID package (version 0.2.2).

2.17 | Hepatic accessibility data

Adult mouse liver accessibility data was published by Chen et al.[36] and the genomic coordinates of the accessible regions of control animals were extracted. Accessible regions near the promoter region of the Nr3c4 (*Ar*) gene were visualised in Integrative Genomics Viewer alongside the GR-bound site assessed by ChIP-qPCR to determine this regions accessibility in untreated conditions.

2.18 | Statistics

Enrichment of AR and GR DNA-binding between subsets of genes was determined by Chi-square tests. Liver lipid levels of the acute cohort were analysed by two-way ANOVAs. One-way ANOVAs were used for the analyses of liver lipid levels of the chronic cohort, as well as for gene expression and CORT levels of the ADX and time series cohort. Tukey multiple comparison tests were used as post-hoc. Direct comparisons between two groups were analysed with unpaired Students t-tests. Statistical analyses were performed with GraphPad Prism 7 software (GraphPad Inc.).

3 | RESULTS

3.1 | Chronic corticosterone-induced transcriptome changes in the liver are partially AR-dependent

To determine the extent of crosstalk between glucocorticoid and androgen signaling in the liver, we performed a transcriptome analysis on the livers of male mice chronically exposed to CORT and CORT combined with AR antagonist ENZA (CORT_ENZA). CORT and CORT_ENZA differentially affected the liver transcriptome compared to vehicle, as is evident from the principal component analysis, rendered as a three-dimensional plot (3D-PCA; Figure 1A). Differential expression analysis revealed extensive transcriptomic changes and identified 3109 differentially expressed genes (DEGs) after CORT (1556 upregulated and 1553 downregulated, Figure 1B) and 3127 DEGs after CORT_ENZA (1701 upregulated and 1426 downregulated, Figure 1C), as compared to vehicle. The effect of co-exposure to ENZA was assessed by analysing CORT_ENZA versus CORT, which revealed a substantial effect of ENZA with 1919 DEGs (1087 upregulated and 832 downregulated after co-exposure to ENZA, Figure 1D).

Visualization of all CORT-regulated genes showed that the effect of chronic CORT on the liver transcriptome was
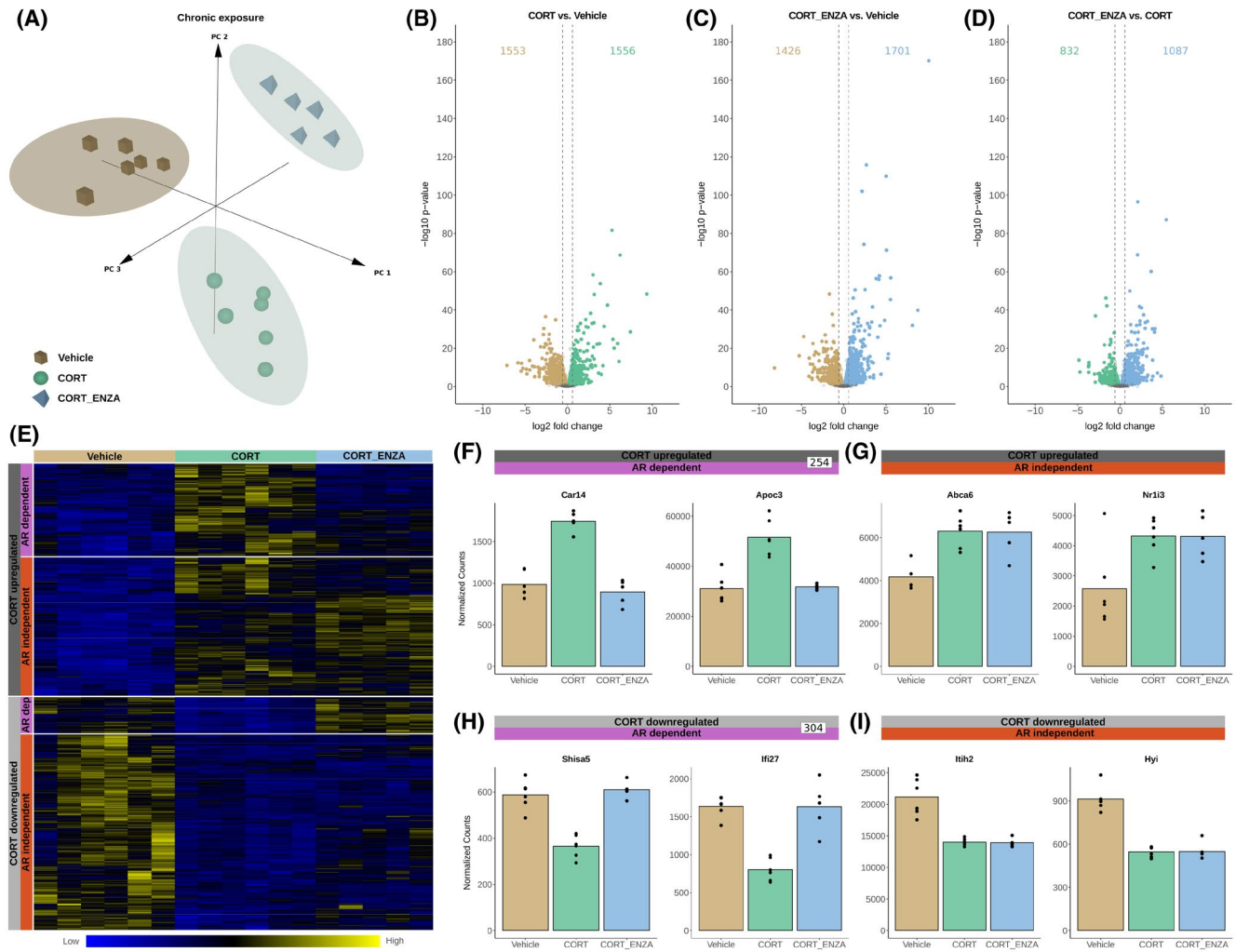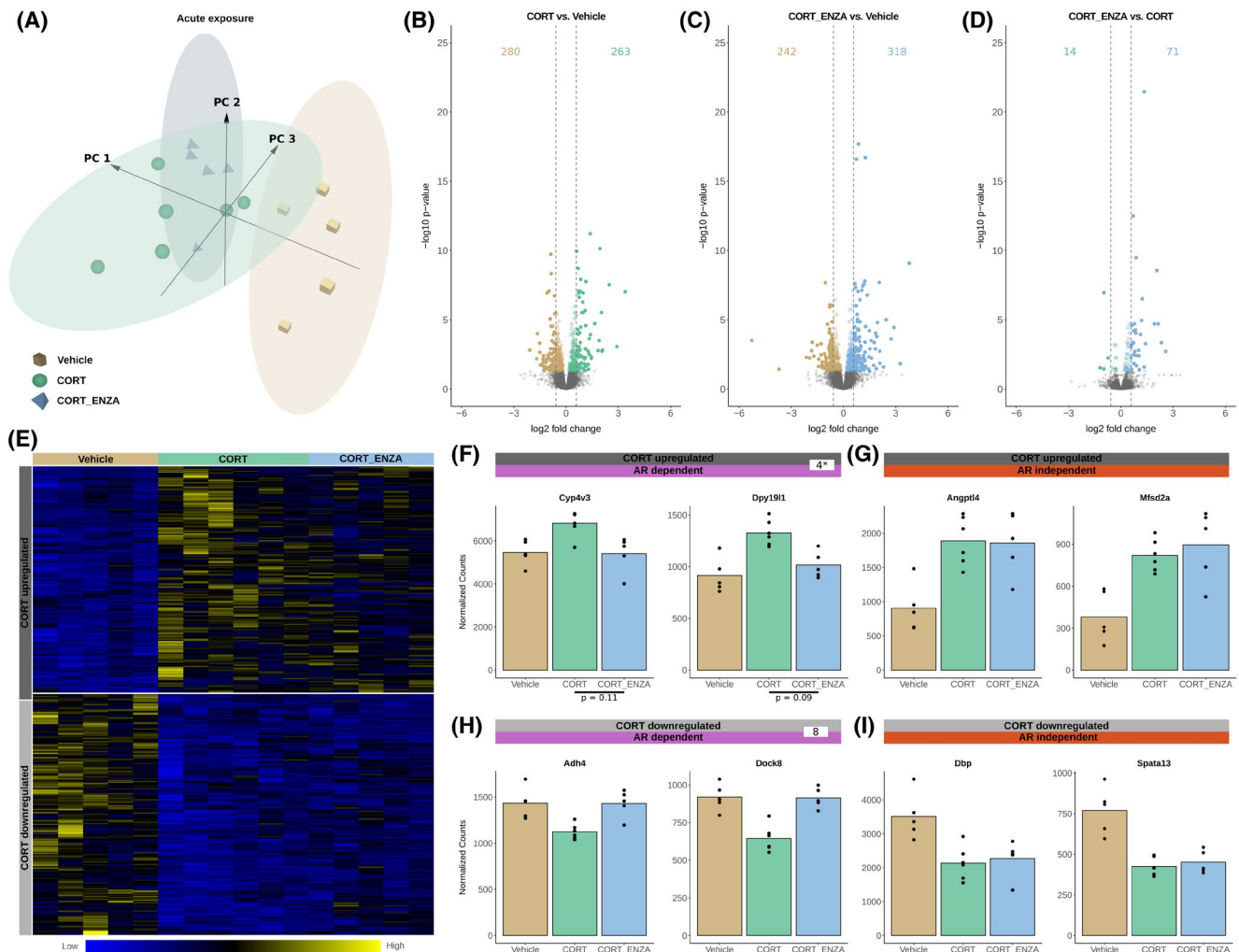
FIGURE 1 The transcriptome after chronic glucocorticoid exposure is partially androgen-dependent in the male mouse liver. (A) Principal component analysis of RNA-seq data of livers obtained from male mice after chronic exposure to vehicle-pellets ($N = 6$), 20 mg corticosterone-pellets (CORT; $N = 6$) and 20 mg corticosterone-pellets + 40 mg/kg/day Enzalutamide (CORT_ENZA; $N = 5$). Volcano-plots of differentially expressed genes between (B) CORT vs. Vehicle, (C) CORT_ENZA vs. Vehicle and (D) CORT_ENZA vs. CORT with the amount of up- and downregulated genes indicated. (E) Heatmap of all genes significantly regulated by CORT, distinguished by hierarchical clustering as CORT upregulated AR dependent, CORT upregulated AR independent, CORT downregulated AR dependent and CORT downregulated AR independent. Example gene expression plots for (F) CORT upregulated AR dependent, (G) CORT upregulated AR independent, (H) CORT downregulated AR dependent and (I) CORT downregulated AR independent. Numbers indicate the total amount of identified AR-dependent CORT-regulated genes

in part lost upon co-exposure with ENZA, revealing an androgen-dependency for a considerable proportion of CORT-regulated genes (Figure 1E). Hierarchical clustering of all CORT-regulated genes identified four classes: (1) CORT-upregulated—AR-dependent (621 genes), (2) CORT-upregulated—AR-independent (930 genes), (3) CORT-downregulated—AR-dependent (242 genes) and (4) CORT-downregulated—AR-independent (1316 genes). Based on this clustering, maximally 28% of all CORT-regulated genes could be classified as AR-dependent. To identify those genes that are strongly AR-dependent, we filtered the clustered genes on significance in the differential expression analysis of the corresponding contrasts.

As such, we classified 254 genes as CORT-upregulated—AR-dependent and 304 genes as CORT-downregulated—AR-dependent. Overall, approximately 18% of all CORT-regulated genes showed robust AR dependence. Representative examples of AR-dependent and AR-independent genes are shown in Figure 1F–I. Our data highlight that chronic glucocorticoid exposure strongly modulates the liver transcriptome, with a subset of genes dependent on AR signaling. However, overlays with available chromatin immunoprecipitation-sequencing (ChIP-seq) data of AR in unstimulated murine liver showed that the set of CORT-regulated—AR-dependent genes was not enriched for AR DNA-binding at any genomic region (e.g.,

FIGURE 2 The transcriptome after acute glucocorticoid exposure is largely androgen-independent in the male mouse liver. (A) Principal component analysis of RNA-seq data of livers obtained from male mice after acute exposure to vehicle ($N = 5$), 3 mg/kg corticosterone for 3 h (CORT; $N = 6$) and 3 mg/kg corticosterone for 3 h after three times 40 mg/kg Enzalutamide exposure at 12 h intervals (CORT_ENZA; $N = 5$). Volcano-plots of differentially expressed genes between (B) CORT vs. Vehicle, (C) CORT_ENZA vs. Vehicle and (D) CORT_ENZA vs. CORT with the amount of up- and downregulated genes indicated. (E) Heatmap of all genes significantly regulated by CORT distinguished by hierarchical clustering as CORT upregulated or CORT downregulated. (F) Example gene expression plots for (F) CORT upregulated AR dependent, (G) CORT upregulated AR independent, (H) CORT downregulated AR dependent and (I) CORT downregulated AR independent. Numbers indicate the total amount of identified AR-dependent CORT-regulated genes. *Adj. $p < 0.15$ CORT_ENZA vs. CORT

promoter, enhancer) compared to the AR-independent genes (33.5% vs. 36.3%, respectively, $x^2(1, N = 504) = 1.2$, p-value $= 0.28$, Table S1).[33] To further address the possible function of co-exposure with ENZA, GO term analysis on the genes differentially expressed between CORT_ENZA and CORT (1919 genes) revealed the cholesterol biosynthetic process. We verified that liver total cholesterol and triglyceride levels were increased in the CORT_ENZA group (Figure S1A–D), confirming functional relevance of the glucocorticoid-androgen crosstalk. Our findings support that glucocorticoids and androgens interact in the mouse liver, but the nature of this interaction requires further investigation.

3.2 | Acute corticosterone-induced transcriptome changes in the liver are largely AR-independent

We next explored how duration of CORT exposure affects glucocorticoid-androgen crosstalk. In a two-by-two experimental setup, we pre-treated mice with either solvent or ENZA for 24 h before subcutaneous injection with vehicle or CORT, 3 h before tissue isolation. We first analysed the same contrasts as applied for the chronic exposure cohort. Based on the 3D-PCA plot, acute exposure to CORT had a distinct effect on the liver transcriptome (Figure 2A), albeit less pronounced as compared to chronic CORT exposure

(Figure 1A). CORT_ENZA did not strongly influence the acute CORT-induced transcriptome. Differential expression analysis revealed 543 DEGs after CORT (263 upregulated and 280 downregulated, Figure 2B) and 560 DEGs after CORT_ENZA (318 upregulated and 242 downregulated, Figure 2C), as compared to vehicle-treated mice. We identified 85 DEGs when comparing CORT_ENZA to CORT (71 upregulated and 14 downregulated, Figure 2D).

Visualization of CORT-regulated genes after acute exposure showed that most of these genes were not androgen-dependent, and hierarchical clustering only identified two clusters associated to transcriptional regulation by CORT (up- and downregulated by CORT; Figure 2E). In an attempt to identify genes with an AR dependency that were not revealed by clustering, we applied the same criteria as used for chronic exposure. This approach identified eight genes that were downregulated by CORT in an AR-dependent manner of which two, *Inf2* and *Per3*, were associated with GR and AR DNA-binding. No CORT upregulated—AR-dependent genes were detected. Increased leniency regarding statistical AR-dependency (adj. *p*-value <0.15 in CORT_ENZA vs. CORT) identified

4 genes displaying the CORT upregulated—AR-dependent expression pattern. Example genes of each identified class are shown (Figure 2F–I). The absence of a clear effect of AR antagonism on the liver transcriptome after acute CORT exposure is in sharp contrast with the sizeable AR-dependent effects after chronic exposure. This indicates that the duration of CORT exposure strongly determined its transcriptional effects in the liver, and its dependence on AR signaling.

3.3 | Duration of corticosterone exposure dictates the effects on the liver transcriptome

In order to find potential clues for the development of AR dependence over time, we investigated the effect duration of CORT exposure had on the liver transcriptome. Acute exposure to CORT resulted in a lower number of DEGs as compared to chronic exposure (543 after acute CORT versus 3109 after chronic CORT, Figure 3A). The percentage of genes associated with GR DNA-binding in
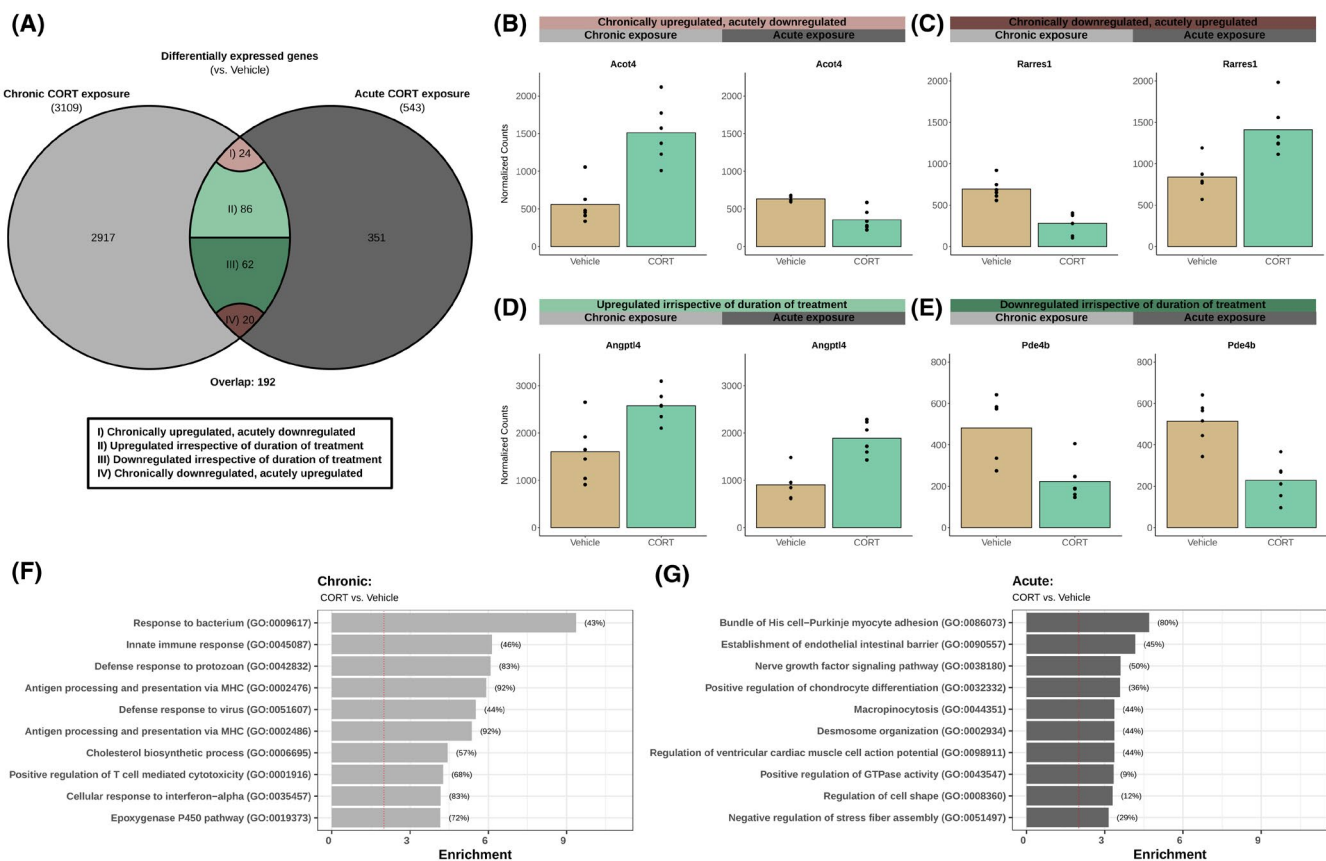
FIGURE 3 Effects of glucocorticoids are strongly dependent on the duration of exposure. (A) Venn-diagram showing the overlap in differentially expressed genes after chronic (14 days) and acute (3 h) exposure to corticosterone (CORT). Example gene expression plots for (B) chronically upregulated, acutely downregulated genes, (C) chronically downregulated, acutely upregulated genes (D) genes upregulated irrespective of duration of exposure and (E) genes downregulated irrespective of duration of exposure. GO term enrichment analysis (top 10) of differentially expressed genes after (F) chronic and (G) acute exposure to CORT. Red-dotted line indicates *p* = 0.01

available ChIP-seq data under high endogenous CORT was significantly higher for the acutely regulated genes in comparison to those regulated after chronic exposure (35% vs. 25% respectively, $x^2(1, N = 977) = 21.6$, p-value <0.0001, Table S1).[32] Only 192 genes were differentially expressed in both conditions (of which 43% was associated to GR DNA-binding). For 44 genes, the directionality of the expression changes differed between the duration of exposure (24 genes were chronically upregulated and acutely downregulated; and 20 genes were chronically downregulated and acutely upregulated; Figure 3B–E). This left 148 genes that were consistently regulated upon acute and chronic CORT exposure.

Given the differences CORT exerted on the liver transcriptome depending on the duration of exposure, a GO term enrichment analysis was performed to compare the biological processes associated with the DEGs (Figure 3F–G). Comparison of the top 10 of enriched biological processes showed no overlap between chronic and acute CORT exposure. This indicated that—besides stronger and more pronounced changes in gene expression upon prolonged CORT exposure—the biological processes affected by CORT differ depending on the duration of exposure. Duration of exposure could contribute to the differences in the extent of AR-dependency found after ENZA co-exposure.

3.4 | Acute transcriptional effects of corticosterone and enzalutamide are predominantly distinct

Given the limited AR dependence of CORT-regulated transcription after acute exposure, we compared the acute effects of CORT and ENZA separately. Hierarchical clustering of the liver transcriptome data showed separation after ENZA exposure from CORT- and vehicle-treated animals (Figure 4A). Differential expression analysis revealed 469 DEGs after ENZA (267 upregulated and 202 downregulated, Figure 4B) compared to vehicle and 1864 DEGs between ENZA and CORT (980 upregulated and 884 downregulated, Figure 4C). Visualization of ENZA-regulated genes showed that most ENZA-upregulated genes were not affected upon acute CORT exposure, while CORT did increase expression levels of a subset of the genes which were downregulated by ENZA (middle cluster, Figure 4D). ENZA and CORT regulated a comparable number of genes (469 after ENZA vs. 543 after CORT), but only 43 genes were differentially expressed by both compounds compared to vehicle (Figure 4E). As transcription of these overlapping genes was regulated by GR activation as well as AR antagonism, these are potential candidates

of direct GR-AR crosstalk. Of these overlapping genes, ten were associated with both GR and AR DNA-binding (*Fbp1*, *Fmo5*, *Gm30505*, *Lrrc42*, *Nr1i2*, *Pard3*, *St6gal1*, *Syne3*, *Usp2*, *Ythdc1*). These genes exhibited the same direction of regulation by CORT and ENZA, which indicates opposite regulatory effects of glucocorticoids and androgens. GO term enrichment analysis for genes regulated by acute CORT exposure did not provide any actionable leads (Figure 3G) and did not overlap with the biological processes enriched by ENZA (Figure S1E), which is in line with the modest overlap in DEGs. The analysis highlighted various metabolic process of which the biosynthesis of cholesterol was most enriched (Figure S1E). Tissue levels of total cholesterol, triglycerides and phospholipids in the liver did not differ after CORT or ENZA exposure at this early timepoint (Figure S1F–H).

3.5 | Glucocorticoids upregulate hepatic AR expression in a GR-dependent manner

We next explored the potential mechanism leading to the observed AR-dependency after chronic CORT exposure, but the lack of AR-dependency in an acute setting. Hepatic *Nr3c4* (*Ar*) mRNA abundance was increased after chronic CORT exposure and not changed after acute exposure (Figure 5A). In parallel with regulation of *Nr3c4* (*Ar*) expression, a portion of coregulators known to interact with AR were differentially expressed after chronic corticosterone exposure (Figure S2A). In addition, the expression of several reported downstream AR-target genes were regulated, suggesting that chronic CORT influences AR signaling (Figure S2B). To further evaluate regulation of AR signaling by glucocorticoids, we performed an experiment in adrenalectomized male C57BL6/J mice implanted with pellets containing different CORT concentrations. This revealed a dose-dependent increase of *Nr3c4* (*Ar*) mRNA expression in the liver as a result of CORT exposure (Figure 5B). We repeated this experiment in intact mice, and similarly found that exposure to 20 mg CORT increased *Nr3c4* (*Ar*) mRNA expression in the liver as compared to vehicle mice (Figure 5C,E). In line with robust changes on *Nr3c4* (*Ar*) mRNA, we confirmed AR upregulation on protein level (Figure 5D). In liver tissue, we observed enrichment of GR DNA-binding to a GRE near the *Nr3c4* (*Ar*) promoter (Figure 5E). This enrichment is likely hepatocyte and EPCAM positive cell specific as only these cell types co-express *NR3C1* (*GR*) and *NR3C4* (*AR*) based on human single-cell liver transcriptomics (Figure S3). Co-immunoprecipitation of GR in livers chronically exposed to CORT showed no direct protein-protein interactions between GR and AR, while
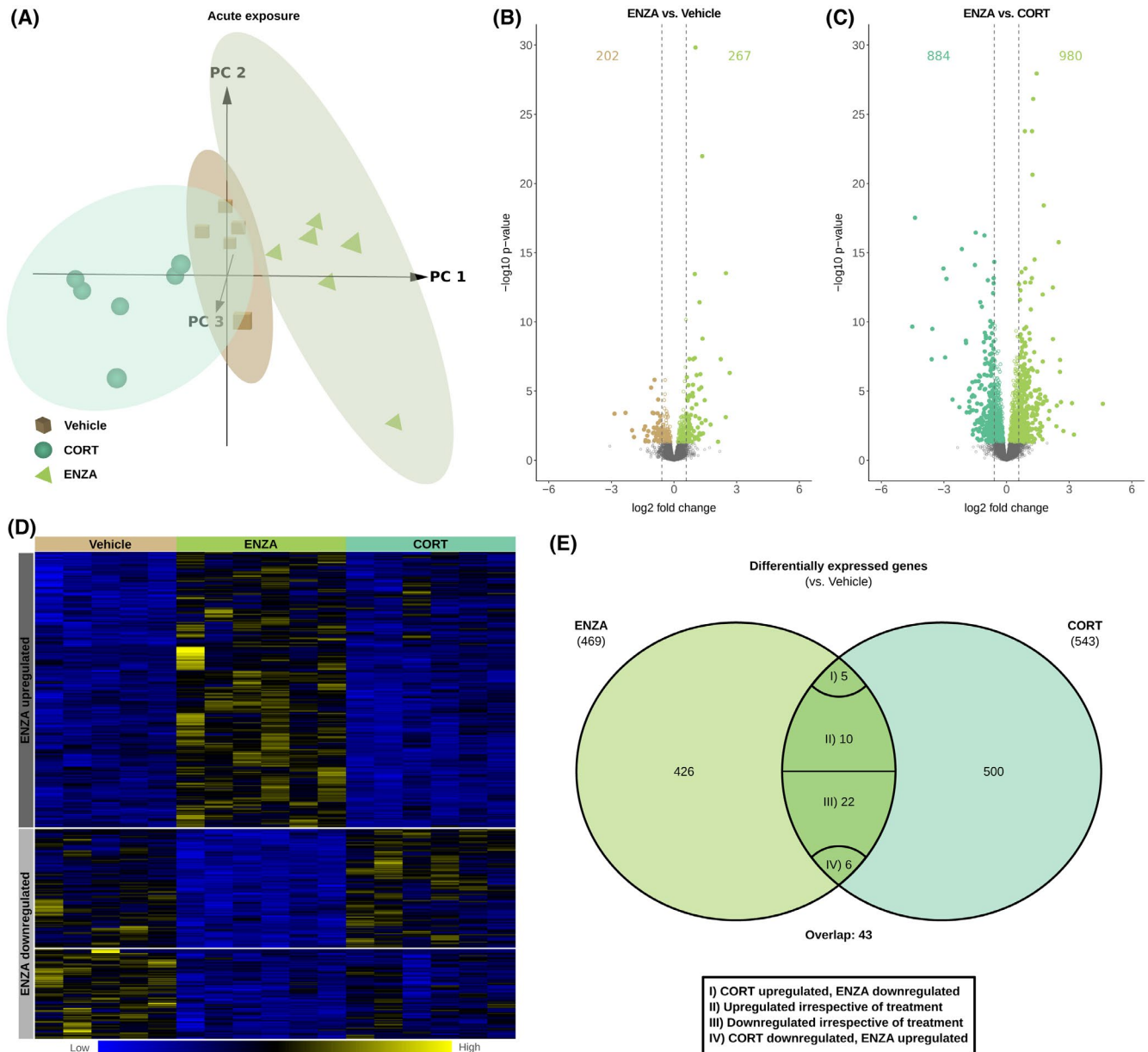
FIGURE 4 Corticosterone and Enzalutamide have distinct effects on the transcriptome in the male mouse liver. (A) Principal component analysis of RNA-seq data of livers obtained from male mice after acute exposure to vehicle ($N = 5$), 3 mg/kg corticosterone for 3 h (CORT; $N = 6$) and 40 mg/kg Enzalutamide (ENZA; $N = 6$). Volcano-plots of differentially expressed genes between (B) ENZA vs. Vehicle and (C) ENZA vs. CORT with the amount of up- and downregulated genes indicated. (D) Heatmap of all genes significantly regulated by Enzalutamide distinguished by hierarchical clustering as ENZA upregulated or ENZA downregulated. (E) Venn-diagram showing the overlap in differentially expressed genes after acute ENZA and CORT exposure

an interaction with a known GR-interactor, UBE3A, was detected (Figure 5F). CORT exposure also upregulated *Nr3c4* (*Ar*) mRNA in white and brown adipose tissue, but not in skeletal muscle (Figure 5G). We further confirmed that AR upregulation was GR-dependent, as the GR specific antagonist Relacorilant[27] significantly reduced *Nr3c4* (*Ar*) mRNA expression after acute dexamethasone and chronic CORT exposure (Figure 5H). Altogether, these results show that glucocorticoids upregulated AR expression, likely direct via a GRE-driven mechanism,

indicating a biological function for AR in glucocorticoid signaling.

3.6 | Glucocorticoid-mediated upregulation of hepatic AR requires prolonged exposure

To further explore the dynamics of AR regulation by glucocorticoids, we performed a time series experiment. Three
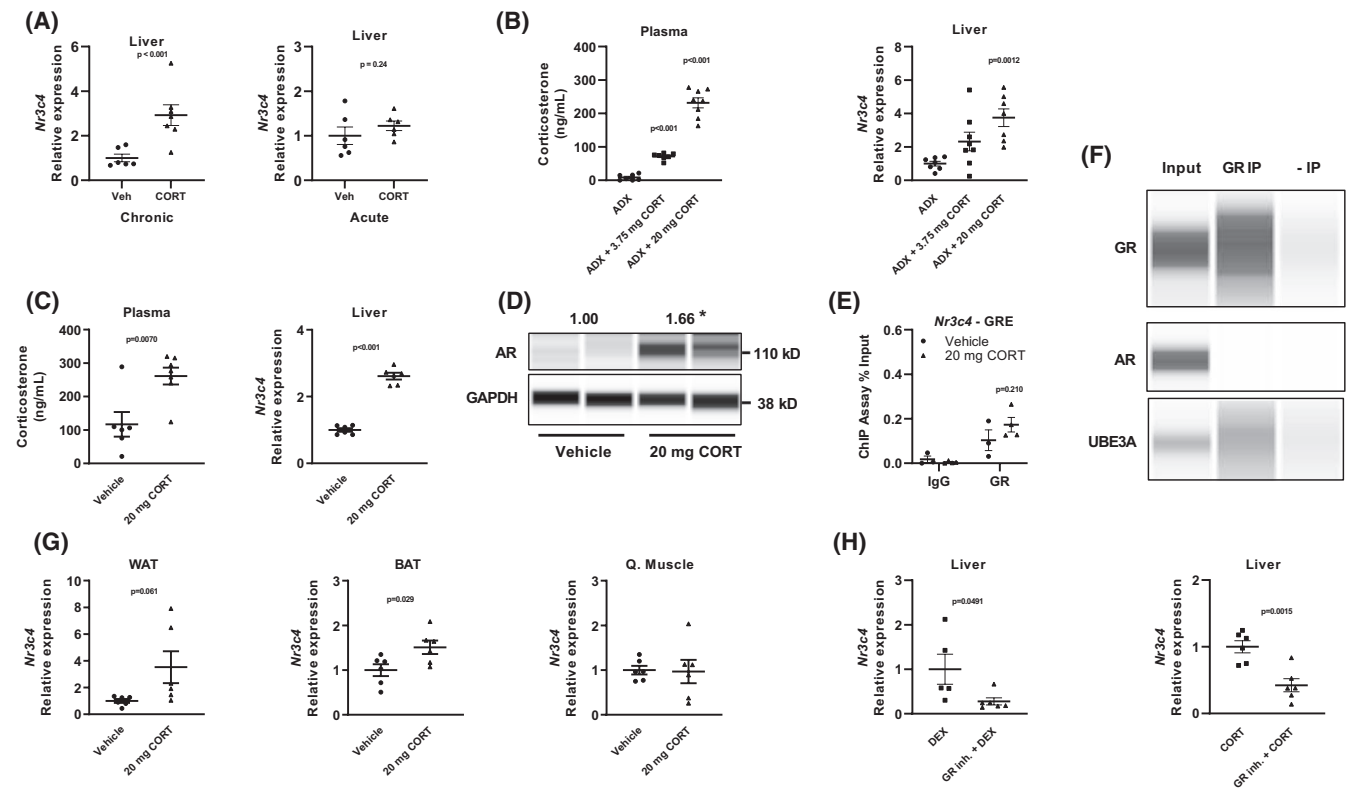
FIGURE 5 Glucocorticoids upregulate hepatic AR expression in a GR-dependent manner. (A) *Nr3c4* (*Ar*) expression in livers of male mice after CORT of the chronic and acute exposure cohorts used for transcriptome analyses. (B) Plasma CORT concentration and *Nr3c4* (*Ar*) expression in livers of adrenalectomized (ADX) male mice implanted with vehicle, 3.75 mg CORT or 20 mg CORT-pellets ($N = 8$). (C) Plasma CORT concentration and *Nr3c4* (*Ar*) expression in livers of intact male mice implanted with vehicle or 20 mg CORT-pellets ($N = 6$). (D) AR protein expression in livers of intact male mice implanted with vehicle or 20 mg CORT-pellets. (E) GR-enrichment at a glucocorticoid response element (GRE) associated with the *Nr3c4* gene ($N = 3$–4). (F) Representative images of co-immunoprecipitation (IP) data of male mice livers exposed to chronic CORT. Input, GR-targeted IP and negative control M2-targeted IP samples analysed for presence of GR, AR and UBE3A in precipitated protein complexes. (G) *Nr3c4* (*Ar*) expression in white adipose tissue (WAT), brown adipose tissue (BAT) and quadriceps muscle (Q. Muscle) of intact male mice implanted with vehicle or 20 mg CORT-pellets ($N = 6$). (H) *Nr3c4* (*Ar*) expression in livers of male mice treated with acute dexamethasone or chronic CORT, with or without GR antagonist Relacorilant ($N = 6$). *$p < 0.05$ vs. Vehicle

hours after injection the plasma CORT levels were not significantly changed, in line with the short plasma half-life of CORT in rodents,[37] while an increase in CORT levels was observed after 6–12 h (Figure 6A). Hepatic *Nr3c1* (*Gr*) mRNA levels were significantly decreased after 3 h, while *Nr3c4* (*Ar*) expression seemed marginally higher after 6 h (Figure 6B,C). AR and GR protein expression levels were not affected by CORT injection (Figure 6D). In livers of control animals, the GRE near the *Ar* promoter was readily accessible, suggesting that the absence of acute CORT effects on *Nr3c4* (*Ar*) expression was likely not due to inaccessibility of the GRE near the *Nr3c4* (*Ar*) gene (Figure 6E).[36] These results show that acute CORT exposure was not sufficient to robustly increase *Nr3c4*/AR expression levels and that prolonged CORT exposure is required for the observed elevation of hepatic *Nr3c4*/AR levels.

4 | DISCUSSION

In this study, we set out to determine the extent of glucocorticoid-androgen crosstalk in the mouse liver. In particular, we studied the effect of glucocorticoid exposure on the liver transcriptome and how this was affected by antagonism of androgen signaling. In adrenally intact male mice, chronic CORT exposure results in robust transcriptomic changes in the liver, of which 18% was dependent on androgen signaling. In sharp contrast, we observed little androgen-dependency after acute exposure to CORT. This suggested that the observed crosstalk may not primarily be supported via direct GR-AR interactions but rather develops gradually, possibly as a metabolic adaptation to chronic stress. Our data show that chronic CORT exposure, but not acute, increases hepatic AR expression, revealing a potential mechanism
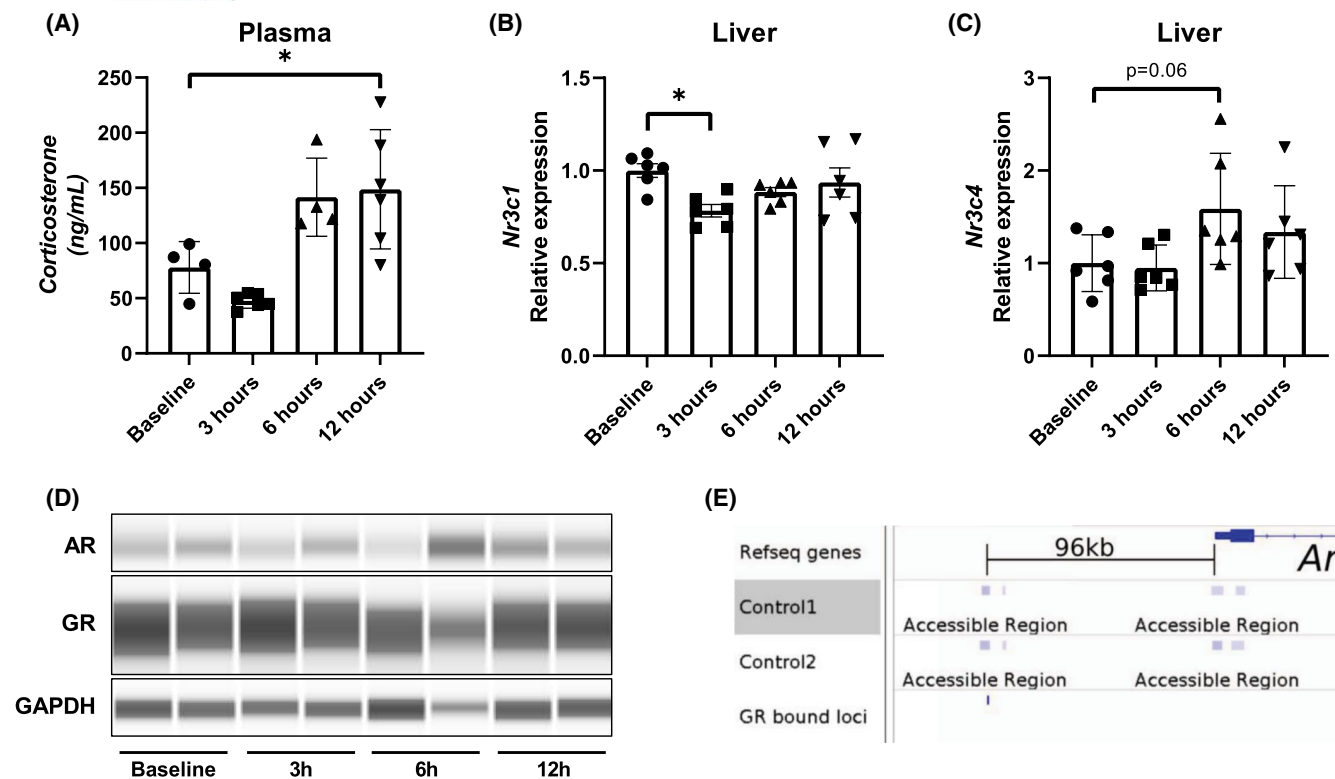
FIGURE 6 Acute glucocorticoid exposure does not strongly affect hepatic AR expression. (A) Plasma CORT concentration of male mice at baseline and 3, 6 or 12 h after injection with 3 mg/kg CORT ($N = 4$–6). (B) *Nr3c1* (*Gr*) and (C) *Nr3c4* (*Ar*) hepatic mRNA expression levels of male mice at baseline and 3, 6 or 12 h after injection with 3 mg/kg CORT ($N = 6$). (D) Representative images of AR, GR and GAPDH protein expression in livers of male mice at baseline and 3, 6, or 12 h after injection with 3 mg/kg CORT. (E) Image adapted from Integrative Genomics Viewer visualising hepatic accessibility data at the AR promoter region and GR bound loci near the *Nr3c4* (*Ar*) gene

and indicating a biological function for the observed AR dependency.

We investigated potential crosstalk between glucocorticoid and androgen signaling by administering CORT combined with the specific AR antagonist ENZA. We chose an AR antagonist over an agonist, as this enabled us to investigate the role of endogenous androgen signaling in glucocorticoid biology. In support of this approach, we previously showed that co-exposure with the AR agonist dihydrotestosterone—on top of endogenous androgens—did not further stimulate the hepatic expression of certain GR responsive genes.[25] RNA-sequencing data showed that 558 out of 3109 DEGs after chronic CORT are androgen-dependent, thereby confirming hepatic transcriptional crosstalk between glucocorticoid and androgen signaling. While previous studies showed that during development many androgen effects in the male liver are mediated by ERα following testosterone conversion to estradiol,[38,39] our current data indicate that in the mature liver of male mice many effects in the context of excessive glucocorticoids are AR-driven.

We tested the hypothesis that direct crosstalk via protein-protein interactions between GR and AR at the genome could underlie androgen dependence of glucocorticoid signaling.[40,41] We predicted that CORT-regulated—AR-dependent genes would be enriched for AR DNA-binding, which we did not observe in a publicly available AR ChIP-seq dataset.[33,42] It is important to note that these livers were obtained from untreated animals with physiological levels of glucocorticoids and androgens—in contrast to our excessive glucocorticoid context—as this can affect AR DNA-binding and therefore influence the transcriptional outcome. The inability to detect enrichment is also in line with the notion that the AR—similarly to the GR—often binds distally from its target genes, which in part impeded the analysis.[43,44] After acute exposure we found that the glucocorticoid-regulated transcriptome in the liver was almost completely AR-independent. AR-dependency after chronic CORT exposure likely originates from indirect crosstalk rather than binding to the same regulatory elements. This is in line with the absence of direct protein-protein interactions between AR and GR in livers exposed to chronic CORT. However, it is remains possible that only after GR-mediated induction, there is sufficient hepatic AR to interact effectively with regulatory elements also utilized by GR.

Multiple modes of indirect crosstalk between nuclear receptors were previously proposed.[13] These modes of

action are challenging to pinpoint as steroid hormone receptors can share downstream target genes, affect the expression of common regulators or regulate genes in the same or converging biological pathways. Comparison of DEGs after 3 h of CORT exposure combined with 24 h of ENZA exposure showed that only 43 genes were regulated by both compounds. This could be an underestimation of the total number of shared target genes as use of an AR agonist in U2OS cells identified 190 shared target genes for AR and GR.[41] The difference may be attributed to the context of the liver tissue and the bone-derived cell line, as cell- and tissue-specific transcriptional effects are in part due to differential expression of coregulators and pioneer factors.[45,46]

In an attempt to better understand the discrepancy between acute and chronic CORT exposure, we directly compared both datasets, and this confirmed that the duration of exposure strongly affected the CORT-regulated transcriptome with more extensive changes after chronic as compared to acute exposure. Of the 192 genes regulated by CORT after both durations of exposure, 44 showed a different directionality, highlighting that the duration of exposure not only determines the regulation of specific gene signatures, but may also shift the direction of regulation.

We found that chronic CORT exposure consistently upregulated hepatic AR expression, both on an mRNA and protein level, likely direct via GR binding near the *Nr3c4* (*Ar*) promoter. In parallel, a number of described AR target genes and AR associated coregulators showed altered expression upon CORT exposure, indicating functional changes in AR signaling. Regulation of AR by glucocorticoids is tissue-specific, with inductions in *Nr3c4* (*Ar*) mRNA expression in white and brown adipose tissue but no effect in quadriceps muscle. Glucocorticoid-driven AR expression was previously reported in a human adipocyte cell line and interpreted as important for adipocyte differentiation, although during this process AR transcriptional activity was suppressed.[47] Of note, we found that baseline AR mRNA and protein levels in the liver were low, even if functional effects of liver-specific AR knockout were previously reported.[21] Altogether, these findings highlight the possibility that glucocorticoids create a dependency on androgen signaling by driving AR expression. Instating this dependency likely occurs via gradual and adaptive changes as acute CORT exposure did not robustly increase hepatic AR expression while its regulatory site is readily accessible for GR. Given that likely only a subset of cells in the liver co-express GR and AR (hepatocytes and EPCAM positive cells), the crosstalk could only occur in these specific cellular subtypes. However, potential involvement of other cell types cannot be excluded, as increased AR expression is observed in hepatic immune cells after chemically-induced liver carcinogenesis.[48] In the current study, we established the acute and chronic effects of glucocorticoids on AR expression in bulk tissue, and while this was sufficient to detect the crosstalk, these data lack the temporal and cellular resolution to fully unravel the precise (possibly cell-type specific) mechanisms,

In physiological conditions, the upregulation of AR in the liver of male mice might be an adaptive strategy for situations that cause chronic exposure to glucocorticoids, but raises the question what happens in females after prolonged glucocorticoid exposure. It has been shown that prolonged glucocorticoid exposure differentially affects males and females, with male mice developing more severe glucocorticoid-induced insulin resistance[49] in an androgen-dependent manner.[50] In addition, the liver transcriptome response of males and females after fasting is vastly different with over 1000 sexually dimorphic genes, a process primed by ERα around birth.[39] Loss of estrogen signaling upon ovariectomy—as a model of menopause—results in a GR-induced steatosis due to follicle stimulating hormone-mediated hyperphosphorylation of the GR.[51] This highlights that crosstalk between sex hormones and glucocorticoids possibly plays a key role throughout various life stages—starting during organ development and persisting in mature tissues and after menopause.

In pathophysiology, androgen and glucocorticoid signaling are associated to the development of NAFLD and its progression towards hepatocellular carcinoma.[23,24] For AR specifically it has been shown that it plays a prominent role in liver carcinogenesis as hepatic AR knockout attenuated tumor development in zebrafish and rodents[52,53] and androgen antagonism inhibited chemically-induced liver carcinogenesis.[48] Our finding that chronic CORT increases hepatic AR levels might contribute to better understand AR-driven liver carcinogenesis and treatment thereof.

We found that the cholesterol biosynthesis pathway is affected after chronic CORT, in accordance with the previously reported increase in plasma cholesterol levels.[25] Co-exposure of chronic CORT with ENZA and acute ENZA exposure alone also point towards cholesterol biosynthesis, suggesting that glucocorticoid and androgen signaling converge at the regulation of lipid metabolism. This notion is consistent with the classical catabolic and anabolic functions of GR and AR, which both require energy availability and redistribution to cope with stressors and build muscle respectively.[54,55] While chronic co-exposure to ENZA decreases plasma cholesterol levels,[25] it results in hepatic cholesterol accumulation. We can speculate that GR upregulates AR signaling to prevent cholesterol accumulation in the liver, possibly by stimulating cholesterol efflux. Such crosstalk is likely not limited to the liver, and further investigation is required to fully elucidate the functional effects of the AR dependency on cholesterol metabolism and potentially other processes.

Our study identified strong differences between acute and chronic glucocorticoid exposure on the liver transcriptome. While it is not exactly known how duration of exposure dictates the effects on the transcriptome, this observation conveys a more general message for other classes of hormones and transcription factors. Our findings clearly stress the importance of carefully considering the duration of treatment, as prolonged exposure may mask the transcriptomic changes that drive functional effects. It also provides a possible explanation for discrepancies between different studies that use different times of exposure. Finally, the differential effects of glucocorticoids after acute and chronic exposure may provide clues on the development of side effects associated with glucocorticoid treatment, and could be involved in treatment insensitivity after long-term drug use.

ACKNOWLEDGMENTS

DISCLOSURES
Authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
Jacobus C. Buurstede, Onno C. Meijer, and Jan Kroon contributed to conceptualization. Methodology: Jacobus C. Buurstede, Susana N. Paul, and Jan Kroon contributed to methodology, investigation and visualization. Onno C. Meijer and Jan Kroon contributed to funding acquisition and supervision. Jacobus C. Buurstede and Jan Kroon contributed to project administration and writing—original draft. Jacobus C. Buurstede, Karolien De Bosscher, Onno C. Meijer, and Jan Kroon contributed to writing—review & editing.

DATA AVAILABILITY STATEMENT
RNAseq data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE180445. Primers used for gene expression analysis are available upon request.

ORCID
Jacobus C. Buurstede 🄳 https://orcid.org/0000-0002-3620-9311

REFERENCES
1. de Quervain D, Schwabe L, Roozendaal B. Stress, glucocorticoids and memory: implications for treating fear-related disorders. *Nat Rev Neurosci*. 2017;18(1):7-19. doi:10.1038/nrn.2016.155

2. Coutinho AE, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol Cell Endocrinol*. 2011;335(1):2-13. doi:10.1016/j.mce.2010.04.005

3. Kadmiel M, Cidlowski JA. Glucocorticoid receptor signaling in health and disease. *Trends Pharmacol Sci*. 2013;34(9):518-530. doi:10.1016/j.tips.2013.07.003

4. Spiga F, Walker JJ, Terry JR, Lightman SL. HPA axis-rhythms. *Compr Physiol*. 2014;4(3):1273-1298. doi:10.1002/cphy.c140003

5. Timmermans S, Souffriau J, Libert C. A General Introduction to Glucocorticoid Biology. *Front Immunol*. 2019;10:1545. doi:10.3389/fimmu.2019.01545

6. Ratman D, Vanden Berghe W, Dejager L, et al. How glucocorticoid receptors modulate the activity of other transcription factors: a scope beyond tethering. *Mol Cell Endocrinol*. 2013;380(1-2):41-54. doi:10.1016/j.mce.2012.12.014

7. Weikum ER, Knuesel MT, Ortlund EA, Yamamoto KR. Glucocorticoid receptor control of transcription: precision and plasticity via allostery. *Nat Rev Mol Cell Biol*. 2017;18(3):159-174. doi:10.1038/nrm.2016.152

8. Whirledge S, DeFranco DB. Glucocorticoid signaling in health and disease: insights from tissue-specific GR knockout mice. *Endocrinology*. 2018;159(1):46-64. doi:10.1210/en.2017-00728

9. Quatrini L, Ugolini S. New insights into the cell- and tissue-specificity of glucocorticoid actions. *Cell Mol Immunol*. 2021;18(2):269-278. doi:10.1038/s41423-020-00526-2

10. Nixon M, Mackenzie SD, Taylor AI, et al. ABCC1 confers tissue-specific sensitivity to cortisol versus corticosterone: a rationale for safer glucocorticoid replacement therapy. *Sci Transl Med*. 2016;8(352):352ra109. doi:10.1126/scitranslmed.aaf9074

11. Draper N, Stewart PM. 11beta-hydroxysteroid dehydrogenase and the pre-receptor regulation of corticosteroid hormone action. *J Endocrinol*. 2005;186(2):251-271. doi:10.1677/joe.1.06019

12. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev*. 2004;25(1):45-71. doi:10.1210/er.2003-0023

13. De Bosscher K, Desmet SJ, Clarisse D, Estebanez-Perpina E, Brunsveld L. Nuclear receptor crosstalk—defining the mechanisms for therapeutic innovation. *Nat Rev Endocrinol*. 2020;16(7):363-377. doi:10.1038/s41574-020-0349-5

14. Presman DM, Ganguly S, Schiltz RL, Johnson TA, Karpova TS, Hager GL. DNA binding triggers tetramerization of the glucocorticoid receptor in live cells, *PNAS*. 2016;113(29):8236-8241. doi:10.1073/pnas.1606774113.

15. Quinn MA, Cidlowski JA. Endogenous hepatic glucocorticoid receptor signaling coordinates sex-biased inflammatory gene expression. *FASEB J*. 2016;30(2):971-982. doi:10.1096/fj.15-278309

16. Duma D, Collins JB, Chou JW, Cidlowski JA. Sexually dimorphic actions of glucocorticoids provide a link to inflammatory diseases with gender differences in prevalence. *Sci Signal*. 2010;3(143):ra74. doi:10.1126/scisignal.2001077

17. Lee I, Kaminski HJ, McPherson T, Feese M, Cutter G. Gender differences in prednisone adverse effects: survey result from the MG registry. *Neurol Neuroimmunol Neuroinflamm*. 2018;5(6):e507. doi:10.1212/NXI.0000000000000507

18. Arora V, Schenkein E, Murali R, et al. Glucocorticoid receptor confers resistance to antiandrogens by bypassing androgen receptor blockade. *Cell*. 2013;155(6):1309-1322. doi:10.1016/j.cell.2013.11.012

19. Yang F, Ma Q, Liu Z, et al. Glucocorticoid Receptor:Megatrans switching mediates the repression of an ERalpha-regulated transcriptional program. *Mol Cell*. 2017;66(3):321-331 e6. doi:10.1016/j.molcel.2017.03.019

20. Bose SK, Hutson I, Harris CA. Hepatic glucocorticoid receptor plays a greater role than adipose GR in metabolic syndrome despite renal compensation. *Endocrinology*. 2016;157(12):4943-4960. doi:10.1210/en.2016-1615

21. Lin H-Y, Yu I-C, Wang R-S, et al. Increased hepatic steatosis and insulin resistance in mice lacking hepatic androgen receptor. *Hepatology*. 2008;47(6):1924-1935. doi:10.1002/hep.22252

22. Balakrishnan M, Patel P, Dunn-Valadez S, et al. Women have a lower risk of nonalcoholic fatty liver disease but a higher risk of progression vs men: a systematic review and meta-analysis. *Clin Gastroenterol Hepatol*. 2021;19(1):61-71.e15. doi:10.1016/j.cgh.2020.04.067

23. Ma WL, Lai HC, Yeh S, Cai X, Chang C. Androgen receptor roles in hepatocellular carcinoma, fatty liver, cirrhosis and hepatitis. *Endocr Relat Cancer*. 2014;21(3):R165-R182. doi:10.1530/ERC-13-0283

24. Woods CP, Hazlehurst JM, Tomlinson JW. Glucocorticoids and non-alcoholic fatty liver disease. *J Steroid Biochem Mol Biol*. 2015;154:94-103. doi:10.1016/j.jsbmb.2015.07.020

25. Spaanderman DCE, Nixon M, Buurstede JC, et al. Androgens modulate glucocorticoid receptor activity in adipose tissue and liver. *J Endocrinol*. 2019;240(1):51-63. doi:10.1530/JOE-18-0503

26. Kroon J, Schilperoort M, In het Panhuis W, et al. A physiological glucocorticoid rhythm is an important regulator of brown adipose tissue function. *Mol Metab*. 2021;47:101179. doi:10.1016/j.molmet.2021.101179

27. Hunt HJ, Belanoff JK, Walters I, et al. Identification of the clinical candidate (R)-(1-(4-fluorophenyl)-6-((1-methyl-1H-pyrazol-4-yl)sulfonyl)-4,4a,5,6,7,8-hexah ydro-1H-pyrazolo[3,4-g]isoquinolin-4a-yl)(4-(trifluoromethyl)pyridin-2-yl)methano ne (CORT125134): a selective glucocorticoid receptor (GR) antagonist. *J Med Chem*. 2017;60(8):3405-3421. doi:10.1021/acs.jmedchem.7b00162

28. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959;37(8):911-917. doi:10.1139/o59-099

29. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550. doi:10.1186/s13059-014-0550-8

30. Zheng DS, Wang X, Antonson P, Gustafsson JA, Li ZY. Genomics of sex hormone receptor signaling in hepatic sexual dimorphism. *Mol Cell Endocrinol*. 2018;471:33-41. doi:10.1016/j.mce.2017.05.025

31. Lempiäinen JK, Niskanen EA, Vuoti K-M, et al. Agonist-specific protein interactomes of glucocorticoid and androgen receptor as revealed by proximity mapping. *Mol Cell Proteomics*. 2017;16(8):1462-1474. doi:10.1074/mcp.M117.067488

32. Hemmer MC, Wierer M, Schachtrup K, et al. E47 modulates hepatic glucocorticoid action. *Nat Commun*. 2019;10(1):306. doi:10.1038/s41467-018-08196-5

33. Li Z, Tuteja G, Schug J, Kaestner KH. Foxa1 and Foxa2 are essential for sexual dimorphism in liver cancer. *Cell*. 2012;148(1-2):72-83. doi:10.1016/j.cell.2011.11.026

34. Heinz S, Benner C, Spann N, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell*. 2010;38(4):576-589. doi:10.1016/j.molcel.2010.05.004

35. Aizarani N, Saviano A, Sagar, Mailly L, et al. A human liver cell atlas reveals heterogeneity and epithelial progenitors, *Nature*. 2019;572(7768):199-204. doi:10.1038/s41586-019-1373-2.

36. Chen TY, Oh S, Gregory S, Shen XL, Diehl AM. Single-cell omics analysis reveals functional diversification of hepatocytes during liver regeneration. *Jci Insight*. 2020;5(22):e141024. doi:10.1172/jci.insight.141024

37. Atkinson HC, Wood SA, Kershaw YM, Bate E, Lightman SL. Diurnal variation in the responsiveness of the hypothalamic-pituitary-adrenal axis of the male rat to noise stress. *J Neuroendocrinol*. 2006;18(7):526-533. doi:10.1111/j.1365-2826.2006.01444.x

38. Della Torre S, Rando G, Meda C, Ciana P, Ottobrini L, Maggi A. Transcriptional activity of oestrogen receptors in the course of embryo development. *J Endocrinol*. 2018;238(3):165-176. doi:10.1530/JOE-18-0003

39. Della Torre S, Mitro N, Meda C, et al. Short-term fasting reveals amino acid metabolism as a major sex-discriminating factor in the liver. *Cell Metab*. 2018;28(2):256-267 e5. doi:10.1016/j.cmet.2018.05.021

40. Claessens F, Joniau S, Helsen C. Comparing the rules of engagement of androgen and glucocorticoid receptors. *Cell Mol Life Sci*. 2017;74(12):2217-2228. doi:10.1007/s00018-017-2467-3

41. Kulik M, Bothe M, Kibar G, et al. Androgen and glucocorticoid receptor direct distinct transcriptional programs by receptor-specific and shared DNA binding sites. *Nucleic Acids Res*. 2021;49(7):3856-3875. doi:10.1093/nar/gkab185

42. Jin HJ, Kim J, Yu J. Androgen receptor genomic regulation. *Transl Androl Urol*. 2013;2(3):157-177. doi:10.3978/j.issn.2223-4683.2013.09.01

43. Bolton EC, So AY, Chaivorapol C, Haqq CM, Li H, Yamamoto KR. Cell- and gene-specific regulation of primary target genes by the androgen receptor. *Genes Dev*. 2007;21(16):2005-2017. doi:10.1101/gad.1564207

44. Wilson S, Qi J, Filipp FV. Refinement of the androgen response element based on ChIP-Seq in androgen-insensitive and androgen-responsive prostate cancer cell lines. *Sci Rep*. 2016;6:32611. doi:10.1038/srep32611

45. Bebermeier J-H, Brooks JD, DePrimo SE, et al. Cell-line and tissue-specific signatures of androgen receptor-coregulator transcription. *J Mol Med (Berl)*. 2006;84(11):919-931. doi:10.1007/s00109-006-0081-1

46. Pihlajamaa P, Sahu B, Lyly L, Aittomaki V, Hautaniemi S, Janne OA. Tissue-specific pioneer factors associate with androgen receptor cistromes and transcription programs. *EMBO J*. 2014;33(4):312-326. doi:10.1002/embj.201385895

47. Hartig S, He B, Newberg J, et al. Feed-forward inhibition of androgen receptor activity by glucocorticoid action in human adipocytes. *Chem Biol*. 2012;19(9):1126-1141. doi:10.1016/j.chembiol.2012.07.020

48. Helms TH, Mullins RD, Thomas-Ahner JM, et al. Inhibition of androgen/AR signaling inhibits diethylnitrosamine (DEN) induced tumour initiation and remodels liver immune cell networks. *Sci Rep*. 2021;11(1):3646. doi:10.1038/s41598-021-82252-x

49. Kaikaew K, Steenbergen J, van Dijk TH, Grefhorst A, Visser JA. Sex difference in corticosterone-induced insulin resistance

in mice. *Endocrinology*. 2019;160(10):2367-2387. doi:10.1210/en.2019-00194

50. Gasparini SJ, Swarbrick MM, Kim S, et al. Androgens sensitise mice to glucocorticoid-induced insulin resistance and fat accumulation. *Diabetologia*. 2019;62(8):1463-1477. doi:10.1007/s00125-019-4887-0

51. Quinn MA, Xu X, Ronfani M, Cidlowski JA. Estrogen deficiency promotes hepatic steatosis via a glucocorticoid receptor-dependent mechanism in mice. *Cell Rep*. 2018;22(10):2690-2701. doi:10.1016/j.celrep.2018.02.041

52. Li HK, Li Y, Lu JW, Huo XJ, Gong ZY. Liver-specific androgen receptor knockout attenuates early liver tumor development in zebrafish. *Sci Rep*. 2019;9:10645. doi:10.1038/s41598-019-46378-3

53. Ma W-L, Hsu C-L, Yeh C-C, et al. Hepatic androgen receptor suppresses hepatocellular carcinoma metastasis through modulation of cell migration and anoikis. *Hepatology*. 2012;56(1):176-185. doi:10.1002/hep.25644

54. Cohen DM, Steger DJ. Nuclear receptor function through genomics: lessons from the glucocorticoid receptor. *Trends Endocrinol Metab*. 2017;28(7):531-540. doi:10.1016/j.tem.2017.04.001

55. Donahue JL, Lowenthal DT. Androgens, anabolic-androgenic steroids, and inhibitors. *Am J Ther*. 2000;7(6):365-373. doi:10.1097/00045391-200007060-00005

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.