Carbonyl reductase 1 amplifies glucocorticoid action in adipose tissue and impairs glucose tolerance in lean mice.

Rachel MB Bell¹, Elisa Villalobos¹, Mark Nixon¹, Allende Miguelez-Crespo¹, Lee Murphy², Angie Fawkes², Audrey Coutts³, Matthew GF Sharp³, Martha V Koerner³, Emma Allan³, Onno C Meijer⁴, René Houtman⁵, Alex Odermatt⁶, Katharina R Beck⁶, Scott G Denham⁷, Patricia Lee⁷, Natalie ZM Homer⁷, Brian R Walker¹,⁸ and Ruth A Morgan¹,⁹*

¹ British Heart Foundation Centre for Cardiovascular Science, The Queen’s Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom. ² Genetics Core, Edinburgh Clinical Research Facility, Western General Hospital, University of Edinburgh, Edinburgh, United Kingdom. ³ Transgenics Core, Bioresearch & Veterinary Services, University of Edinburgh, Edinburgh, United Kingdom. ⁴ Department of Internal Medicine, Division of Endocrinology, Leiden University Medical Center, Leiden, The Netherlands ⁵ Pamgene International, Den Bosch, The Netherlands, ⁶ Division of Molecular and Systems Toxicology, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland. ⁷ Mass Spectrometry Core Laboratory, Wellcome Trust Clinical Research Facility, The Queen’s Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom ⁸ Clinical and Translational Research Institute, Newcastle University, Newcastle upon Tyne, United Kingdom, ⁹ Royal (Dick) School of Veterinary Studies, University of Edinburgh, Midlothian, United Kingdom.

*Corresponding author. University of Edinburgh, Centre for Cardiovascular Science, The Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh, EH14 6TJ. Phone: 07739079919 ruth.morgan@ed.ac.uk

Emails: rachel.bell@ed.ac.uk (R.M.B. Bell), evillalo@exseed.ed.ac.uk (E. Villalobos), m.nixon@ed.ac.uk (M. Nixon), amiguele@exseed.ed.ac.uk (A. Miguelez), lee.murphy@ed.ac.uk (L. Murphy), a.fawkes@ed.ac.uk (A. Fawkes), aduncan5@exceed.ed.ac.uk (A. Coutts), matthew.sharp@ed.ac.uk (M.G.F. Sharp), m.koerner@ed.ac.uk (M.V. Koerner), emma.murdoch@igmm.ed.ac.uk (E. Allan), o.c.meijer@lumc.nl (O.C. Meijer), rene@precisionmedicinelab.nl (R. Houtman), alex.odermatt@unibas.ch (A. Odermatt), katharina.beck@wlab.gu.se (K.R. Beck), scott.denham@ed.ac.uk (S.G. Denham), t.lee@exceed.ed.ac.uk (T. Lee), n.z.m.homer@ed.ac.uk (N.Z.M. Homer), brian.walker@newcastle.ac.uk (B.R. Walker), ruth.morgan@ed.ac.uk (R.A. Morgan).

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Author Contributions:

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Highlights

- Carbonyl reductase 1 (Cbr1) is a novel regulator of adipose glucocorticoid metabolism and glucose homeostasis.
- Cbr1 metabolises corticosterone to 20β-dihydrocorticosterone which is both a glucocorticoid and mineralocorticoid receptor agonist.
- Global knockdown of Cbr1 improves glucose tolerance in lean male mice.
- Overexpression of Cbr1 in adipose tissue worsens glucose tolerance in lean male and female mice.

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Abbreviations: Cbr1, Carbonyl reductase 1; LC-MS/MS, liquid chromatography tandem mass spectrometry; 20β-DHB/F, 20β-dihydrocorticosterone/dihydrocortisol; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; SNPs, single nucleotide polymorphisms; PuroR, Puromycin resistance; UTR, untranslated region; Tk, Thymidine kinase; NeoR, neomycin resistant; RMCE, Recombination-Mediated Cassette Exchange; NEFA, non-esterified fatty acids; GTT, glucose tolerance test; ITT, insulin tolerance test; ELISA, enzyme linked immunoassay; qPCR, quantitative polymerase chain reaction; 11β-HSD1/2, 11β-hydroxysteroid dehydrogenase type 1/2; DEGs, differentially expressed genes.

Keywords: Obesity, glucocorticoid, metabolism, glucose, corticosterone, mineralocorticoid receptor, glucocorticoid receptor.
Abstract

Objective:
Carbonyl reductase 1 (Cbr1), a recently discovered contributor to tissue glucocorticoid metabolism converting corticosterone to 20β-dihydrocorticosterone (20β-DHB), is upregulated in adipose tissue of obese humans and mice and may contribute to the cardiometabolic complications of obesity. This study tested the hypothesis that Cbr1-mediated glucocorticoid metabolism influences glucocorticoid and mineralocorticoid receptor activation in adipose tissue, and impacts glucose homeostasis in the lean and obese state.

Methods:
The actions of 20β-DHB on corticosteroid receptors in adipose tissue were investigated first using a combination of in silico, in vitro and transcriptomic techniques and then in vivo administration in combination with receptor antagonists. Mice lacking one Cbr1 allele and mice overexpressing Cbr1 in their adipose tissue underwent metabolic phenotyping before and after induction of obesity with high-fat feeding.

Results:
20β-DHB activated both the glucocorticoid and mineralocorticoid receptor in adipose tissue and systemic administration to wild-type mice induced glucose intolerance, an effect which was ameliorated by both glucocorticoid and mineralocorticoid receptor antagonism. Cbr1 haploinsufficient lean male mice had lower fasting glucose and improved glucose tolerance compared with littermate controls, a difference that was abolished by administration of 20β-DHB and absent in female mice with higher baseline adipose 20β-DHB concentrations than male mice. Conversely, overexpression of Cbr1 in adipose tissue resulted in worsened glucose tolerance and higher fasting glucose in lean male and female mice. However, neither Cbr1 haploinsufficiency nor adipose overexpression affected glucose dyshomeostasis induced by high-fat feeding.

Conclusions:
Carbonyl reductase 1 is a novel regulator of glucocorticoid and mineralocorticoid receptor activation in adipose tissue which influences glucose homeostasis in lean mice.
1. Introduction

Glucocorticoids act through widely expressed glucocorticoid receptors (GR) and the more tissue-specific mineralocorticoid receptor (MR) to modulate fuel metabolism, the immune system and salt and water balance. Adipose tissue expresses both GR and MR, with the balance of activation between the two being an important determinant of adipose tissue expansion, insulin sensitivity and glucose homeostasis [1] [2]. Excessive or chronic activation of GR and/or MR in adipose tissue results in glucose intolerance and lipid accumulation and contributes to metabolic syndrome [3-7]. Glucocorticoids are the main ligands of GR, whilst MR binds both glucocorticoids and aldosterone. In classic MR-responsive tissues, such as the kidney, aldosterone binding is favoured due to the presence of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) which inactivates cortisol/corticosterone. In adipose tissue, however, there is little 11β-HSD2 and glucocorticoids are the primary ligands of MR [8]. Receptor activation by glucocorticoids in adipose tissue is modulated by steroid-metabolising enzymes, such as 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and 5α-reductases, which catalyse the conversion of primary glucocorticoids into more or less potent ligands of the receptors [9; 10]. Dysregulation of these glucocorticoid-metabolising enzymes in adipose tissue directly contributes to insulin dysregulation [11; 12] and can contribute to the pathogenesis of obesity and cardiovascular disease [9; 13; 14].

We recently showed that the cytosolic enzyme Carbonyl Reductase 1 (Cbr1) is a novel regulator of tissue glucocorticoid metabolism, converting cortisol/corticosterone into 20β-dihydrocortisol (20β-DHF) or 20β-corticosterone (20β-DHB), both of which are weak agonists of the human and murine GR [15]. Cbr1 and 20β-DHF/B are abundant in adipose tissue and increased in obese adipose of humans and mice [15; 16]. There is growing evidence that Cbr1 can affect metabolism and in particular glucose homeostasis. Single nucleotide polymorphisms (SNPs) in the human CBR1 gene which increase CBR1 expression are causally associated with higher fasting blood glucose [15] and deficiency of the key transcriptional regulator of Cbr1, Nrf2, improves glucose tolerance in murine models [17]. Cbr1 was identified by RNA-sequencing as a key gene involved in the pathogenesis of a streptozotocin-induced rat model of diabetes [18] and is significantly upregulated in the rat heart in diabetes [19]. Reduced CBR1 may contribute to the metabolic benefits of a Mediterranean diet since polyphenol constituents are inhibitors of CBR1 [20; 21]. Despite this evidence, there have not been any definitive intervention studies to test the role of CBR1 in metabolic health.

Having previously shown that 20β-DHF/B activates GR, we first tested the hypothesis that 20β-DHB is a ligand of MR in adipose tissue and that its administration impairs systemic glucose tolerance through increased GR and MR activation. We then addressed the hypothesis that global deficiency of Cbr1 reduces plasma and tissue 20β-DHB, resulting in a reduction in GR and MR activation and improved glucose tolerance with or without high fat feeding. Finally, we tested the hypothesis that metabolic effects of Cbr1 are mediated in adipose tissue by generating a model of adipose-specific overexpression.

2. Methods

2.1 Animals

Animal experiments were approved by the University of Edinburgh ethical committee and performed under the Provisions of the Animals Scientific Procedures Act (1986) of the UK Home Office, in accordance with EU Directive 2010/63/EU.

Mice heterozygous for Cbr1 deletion were generated; homozygosity of this gene deletion is foetal lethal [22]. A targeting vector was designed to introduce loxP sequences flanking Cbr1 exons 2 and 3 (including the 3’ untranslated region) (Taconic Biosciences GmBH, Leverkusen, Germany). The positive selection marker (Puromycin resistance- PuroR) was flanked by F3 sites and inserted downstream of the 3’ UTR. The targeting vector was generated using BAC clones from the C57BL/6J
RPCI-23 BAC library and were transfected into the Taconic Biosciences C57BL/6NTac embryonic stem cell line. Homologous recombinant clones were isolated using positive (PuroR) and negative (Thymidine kinase- Tk) selections. The constitutive knock-out allele was obtained by treating 1-cell embryos with soluble HTN-Cre enzyme (Excellgen, Rockville, MD, USA) as described previously [23; 24]. Mice were genotyped by Transnetyx (Memphis, TN, USA) using real-time qPCR (RT-qPCR).

To generate adipose specific over-expressors of Cbr1 animals with floxed Cbr1 (R26-Cbr1FL) were first generated (Taconic Biosciences GmBH). The following elements were inserted into the Rosa26 locus using Recombination-Mediated Cassette Exchange (RMCE): a CAG promoter cassette, a loxP-flanked transcription termination cassette (STOP) containing a combination of polyadenylation signals, the Cbr1-T2A-mKate2 open reading frame together with a Kozak sequence (GCCACC), the human Growth Hormone (hGH) polyadenylation signal and an additional polyadenylation signal. The RMCE vector was transfected into the Taconic Biosciences C57BL/6 ES cell line equipped with RMCE docking sites in the ROSA26 locus. The recombinant clones were isolated using positive (Neomycin resistance- NeoR) selection. The adipose-specific over-expressors (R26-Cbr1Adpq) were obtained by crossing with Adiponectin-Cre mice [25]. Mice were genotyped by Transnetyx, using RT-qPCR.

Male and female mice were maintained in individual ventilated cages at 21°C on a 12-h light/12-h dark cycle with free access to food and water unless otherwise stated. Mice were given a high-fat diet (D12331, Research Diets Inc., NJ, USA) for 8 weeks. Bodyweight and food intake were measured weekly using a precision scale. Body composition was determined using time-domain nuclear magnetic resonance (Bruker, Billerica, MA, USA) before and after high-fat feeding. Mice undergoing adrenalectomy were maintained on 0.9% saline. Blood for glucocorticoid analysis was collected following decapitation between 9am and 10am, the animals were not fasted.

2.2 Extraction and quantification of mRNA by RT-qPCR

Total RNA was extracted from adipose and liver using the RNAeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to manufacturer’s instructions. The tissue was mechanically disrupted in either QIAzol (Qiagen) for adipose tissue or RLT buffer (Qiagen) for liver tissue. cDNA was synthesised using QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction was performed using a Light-cycler 480 (Roche Applied Science, Indianapolis, IN, USA). Primers were designed using sequences from the National Centre of Biotechnological Information and the Roche Universal Probe Library. The qPCR primer sequences are included in Supplementary Table 1. Samples were analysed in triplicate and amplification curves plotted (y axis, fluorescence; x axis, cycle number). Triplicates were deemed acceptable if the standard deviation of the crossing point was < 0.5 cycles. A standard curve (y axis, crossing point; x axis, log concentration) for each gene was generated by serial dilution of cDNA pooled from different samples, fitted with a straight line and deemed acceptable if reaction efficiency was between 1.7 and 2.1. The average of housekeeping genes 18s, Tbp and β-actin was used to normalise gene expression.

2.3 Quantification of protein by Western Blot

Protein lysates from subcutaneous adipose tissue and liver (30-50 mg) were prepared in RIPA lysis buffer supplemented with protease inhibitors (Thermo Scientific™, Waltham, MA, USA). Protein concentration was quantified using bicinechonic acid (BCA) assay (Thermo Scientific™). Extracted proteins (20 μg) were resolved by SDS-PAGE, using Criterion TGX Precast Protein Gels 4-20% (Bio-Rad) under reducing and denaturing conditions. Proteins were transferred to nitrocellulose membranes using Trans-Blot® Turbo™ Blotting System (Bio-Rad). Membranes blocked with skimmed milk at 5% in Tris-buffered saline, and then subjected to western blotting using antibodies
specific for CBR1 (rabbit polyclonal IgG, cat. n° NBP1-86595, Novus Biologicals), β-Actin (mouse monoclonal IgM, 7D2C10, cat. n° 60008-1-Ig, Proteintech). The primary antibodies were used at 1:1000 and 1:5000 dilution in 3% BSA in Tris-buffered saline and incubated overnight (4°C). Secondary antibodies IRDye 800CW or IRDye 680CW (LI-COR) (anti-mouse and rabbit IgGs) were used at 1:10000 dilution in 3% BSA in Tris-buffered saline and incubated for 1 hour at room temperature. Protein detection was performed using an Odyssey CLx Imaging system (LI-COR). Densitometric analyses were performed using Image Studio™ Software (LI-COR).

2.4 CBR1 activity

CBR1 activity was determined in adipose and liver homogenised in Kreb’s buffer as previously described [26]. Briefly, homogenates (1 mg/ml protein) were incubated with menadione (100 nM) and NADPH (2 mM) and the absorbance measured at 340 nm at 25°C for 45 minutes using a Microplate reader Spectra Max Plus (Molecular Devices, LLC). Activity was defined as (ΔAbs340/min)/6.3 and expressed per mg protein.

2.5 Quantification of steroids in plasma and adipose by LC-MS/MS

Mouse plasma (100 µL) samples were prepared alongside calibration standards (covering the range 0.025-500 ng/mL) in a 96-well plate, enriched with internal standard (10 ng, d8-corticosterone, d8-aldosterone) and diluted with 0.1% formic acid in water (100 µL) on a Biotage® Extrahera™ liquid handling robot. Diluted samples were transferred to an SLE+ 200 plate and eluted into a collection plate with dichloromethane/propan-2-ol (98:2; 4 x 450 µL). The eluate was dried down and reconstituted in water/methanol (70:30, 100 µL) before injecting directly from the 96-well plate for LC-MS/MS analysis.

Adipose tissue samples (60-80 mg) were enriched with internal standard (0.5 ng; d8-corticosterone) homogenized (TissueLyser II, Qiagen) in acetonitrile w/ 0.1% formic acid (500 µL). A calibration standard curve of 20β-DHB was prepared alongside the samples, covering the range 0.0025-10 ng. Samples were centrifuged and the supernatant (500 µL) transferred to an ISOLUTE® PLD+ 96-well plate cartridge (Biotage, Uppsala, Sweden), subjected to positive pressure and collected and dried down under nitrogen gas (40°C). Samples were re-suspended in H2O: MeOH (70:30; 100 µL), sealed before analysis.

Extracts were analysed by LC-MS/MS on a Shimadzu Nexera X2 connected to a QTrap 6500+ mass spectrometer (AB Sciex,), adapted from earlier methods [27]. Standards and samples were injected (20 µL) on to a Kinetex C18 column (100 x 3.0 mm; 2.6 µm; Phenomenex, UK) fitted with a 0.5 µm Ultra KrudKatcher (Phenomenex), using a mobile phase system of A - 0.05 mM ammonium fluoride in water and methanol at 0.5 mL/min from 50-90% corticosterone over 16 mins. Mass transitions and retention times are detailed in supplementary materials. Peaks were integrated using Quantitate software and the peak area ratio of 20β-DHB to d8-corticosterone using least squares regression with 1/x weighting was used to calculate the amount of 20β-DHB in the samples, then normalized to the tissue weight, expressed as ng/g of tissue.

2.6 Glucose and insulin tolerance tests

For glucose tolerance tests, mice were fasted for 6 h (0800-1400h) in clean cages and then given glucose (2 mg/g bodyweight, 40% w/v in saline) via intraperitoneal injection. For insulin tolerance tests, mice were fasted for 4 h (1000-1400h) and then administered 0.75 IU/kg insulin (Eli Lilly, IN, USA) via intraperitoneal injection. Blood was collected from the tail vein immediately prior to and 15, 30, 60, 90 and 120 minutes after injection. Glucose was measured immediately using a point-of-care glucometer (Accu-Chek Aviva, Roche, Basel, Switzerland). Plasma insulin was measured using the Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem Inc., IL, USA). Plasma non-esterified
fatty acids were measured by ELISA (NEFA-HR, Wako Chemicals GmBH, Neuss, Germany), following the manufacturer’s instructions.

### 2.7 20β-DHF interaction with human mineralocorticoid receptor

Docking studies were performed using the GOLD software version 5.2 (Cambridge Crystallographic Data Centre, Cambridge, UK) [28]. This software allows the identification of precise docking poses for small molecules in the binding pocket of a protein applying a genetic algorithm. The crystal structures with the Protein Data Bank (PDB) entry 2AA2 [DOI: 10.2210/pdb2AA2/pdb] was selected for MR. First the respective co-crystallized ligand, aldosterone, was removed from the binding pocket and re-docked into the binding site to examine whether GOLD could restore the original binding position and therefore validate the docking settings. The MR binding sites were defined by the ligand surrounded by a 6 Å region lining the active site. Protein ligand interactions determined by the docking software were further assessed using LigandScout 3.12 (inte:ligand GmbH, Vienna, Austria, kindly provided by Thierry Langer). Based on chemical functionalities, geometric distances and angles between adjacent structures, this software automatically evaluates the observed binding pattern between the protein and the docked ligand [29]. A microarray Assay for Real-time Co-regulator-Nuclear Receptor Interaction (MARCoNI) was used to compare the quantitative and qualitative co-regulator recruitment induced when 20β-DHF (1 μM) binds with human MR with that of recruitment in response to aldosterone (1 μM) using a previously described method [30].

### 2.8 In vitro mineralocorticoid receptor activation

The human embryonic kidney cell line, HEK293 cells were obtained from the European Collection of Cell cultures (ECACC; distributor Sigma-Aldrich, St-Louis, MO, USA). Cells were grown and maintained in a humidified atmosphere (95% air, 5% CO₂, 37°C) in Dulbecco’s modified Eagle’s medium (DMEM, Lonza Group Ltd., Basel, Switzerland) supplemented with glucose (4.5 g/L), heat-inactivated fetal bovine serum (HI-FBS) (10% v/v), penicillin (100 IU/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM). Cells were seeded at 2 x 10^5 per 35-mm well. Cells were rinsed twice with PBS and cultured in steroid-free medium for 24 h prior to experimentation. Cells were transiently transfected with 1μg of pMMTV LTR–luciferase, 1μg of pKC275 (encoding β-galactosidase as internal control) and 0.05 μg of murine MR. Cells were treated with vehicle (ethanol), aldosterone (10^{-14}-10^{-5} M, Sigma-Aldrich) or 20β-DHB (10^{-12}-10^{-5} M, Steraloids, Newport, RI, USA) for 4 hours and were then lysed and luciferase and β-galactosidase activities measured as described previously [31]. Galactosidase activity was assayed using a Tropix Kit (Applied Biosystems, Foster City, CA, USA). The mean ratio of luciferase/β-galactosidase activities was calculated. Plasmids were a kind gift from K.E. Chapman, Centre for Cardiovascular Science, University of Edinburgh.

### 2.9 RNA sequencing analysis of adipose tissue

C57BL/6J male mice (8 weeks of age n= 6/group) underwent adrenalectomy to remove endogenous steroids as previously described [32]. Seven days post-surgery subcutaneous mini-osmotic pumps (Alzet® Cuppertino, CA, USA) were implanted to deliver either vehicle (DMSO/Propylene glycol), the GR agonist dexamethasone, the MR agonist aldosterone or 20β-DHB (20 μg/day). After 7 days of treatment subcutaneous adipose tissue was harvested at post-mortem and RNA extracted as described previously (Section 2.4). Total RNA samples were quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc.) and the Qubit RNA HS assay kit. RNA integrity was assessed using the Agilent 2100 Bioanlyser System (Agilent Technologies Inc.) and Agilent RNA 6000 Nano kit. Libraries were prepared from 500 ng of each total-RNA sample using the TruSeq Stranded mRNA Library Kit (Illumina Inc.). cDNA was synthised and libraries quantified, these and details of next generation sequencing are given in supplementary material.

### 2.10 Statistical analysis
Analyses were performed using Prism 8 for software (Graph-Pad, San Diego, CA, USA). All variables were assessed for normality using the Kolmogorov-Smirnov test. Comparisons between groups were performed using Student's t-tests or Mann-Whitney test, as appropriate. Comparisons between groups at different time points and between >2 groups were assessed by one or two-way ANOVA, with Bonferroni post-hoc. Data are presented as mean ± SEM.
3. Results

3.1 20β-DHF/B is full mineralocorticoid receptor agonist in vitro

As there is no crystal structure of murine MR available, in silico modelling of 20β-DHF, the human equivalent to 20β-DHB, was conducted on the human MR. Docking calculations revealed similar interactions with the residues of the MR ligand binding pocket for 20β-DHF and aldosterone. Both ligands formed hydrogen (H)-bonds with Gln776, Asn770 and Thr945. The 20β-hydroxyl group on 20β-DHF was found to form an H-bond with Met845, whereas the carbonyl group of aldosterone at the same position showed an H-bond with Cys942 (Figure 1A). Based on this, we predicted that upon binding to the receptor 20β-DHF was likely to induce a transcriptional response. This was tested in vitro using HEK293 cells transiently expressing murine MR and a luciferase reporter under the control of a promoter with a corticosteroid receptor response element. A dose response curve showed that 20β-DHB could fully activate murine MR (20β-DHB EC50 8.5 x 10^{-8} M versus aldosterone EC50 5.7 x 10^{-11}) (Figure 1B), and to a lesser extent murine GR (partial agonism at EC50 2.5 x 10^{-6} [15]). Thus, 20β-DHB is a more potent agonist of MR than of GR in vitro.

On binding a ligand, the translocation to the nucleus and subsequent transcriptional response to a steroid hormone-receptor complex is largely determined by co-regulator recruitment [30]. We have previously shown that on binding to GR 20β-DHF recruits only 36% of those co-regulators recruited by cortisol [15]. On testing the effect of 20β-DHF on MR we found that 20β-DHF-MR binding recruited 93% of the co-regulators recruited by the aldosterone-MR complex or the cortisol-MR complex (Figure 1C-D, Supplementary File 1) and recruitment by both ligands was highly correlated (R^2 = 0.97, p < 0.0001) (Figure 1E-G), that is to say that all of the co-regulators were recruited in the same direction by both ligands and it was only the magnitude of fold change that differed between the two. This is in line with the observed full agonism seen in the transactivation assay.
3.2 20β-DHB induces GR and MR-mediated transcriptional and functional responses in vivo

These in vitro findings suggest that 20β-DHB would preferentially activate MR over GR in vivo. To determine the relative activation of GR and MR by 20β-DHB we compared the transcriptional response of subcutaneous adipose tissue to systemic infusion of either the GR agonist dexamethasone, the MR agonist aldosterone or 20β-DHB (20 μg/day of each agonist) in wild-type mice (C57BL/6J), following adrenalectomy to remove endogenous steroids. Candidate gene expression analysis showed that dexamethasone and 20β-DHB induced transcription of candidate GR-responsive gene Pnpla2 and mixed GR/MR target gene Tsc22d3 in subcutaneous adipose tissue (Figure 2A). Unlike dexamethasone, 20β-DHB did not downregulate GR or Tnfa (Figure 2A). Expression of MR-responsive gene Ptgds was increased by aldosterone and 20β-DHB (Figure 2A). Given the rarity of known MR-specific genes, we further explored the transcriptome induced by each ligand using next generation sequencing of the subcutaneous adipose tissue.

20β-DHB differentially regulated 1039 genes (554 up-regulated, 485 down-regulated, Supplementary File 2), dexamethasone 8782 genes (3939 up-regulated, 4843 down-regulated) and aldosterone 855 (303 up-regulated, 552 down-regulated) (Figure 2B-D) compared with vehicle control (DMSO). 20β-DHB shared 54% of its differentially expressed genes (DEGs) with dexamethasone but this accounted
for just 5% of the genes regulated by dexamethasone (561/8782), 15% with aldosterone accounting for 18% of aldosterone regulated genes (155/855) and 8% with both; 24% were uniquely regulated by 20β-DHB. We further analysed these genes for the presence of conserved GR binding sites using oPOSSUM software [33]. Within the genes uniquely differentially regulated by 20β-DHB, 10% (26/252) had distinct GR transcription factor binding sites (TFBS). This was similar to, but slightly less than, the percentage of TFBS identified in the genes uniquely regulated by dexamethasone (12%, 814/6414) and aldosterone (15%, 50/315). Transcriptome interrogation of the shared DEGs by KEGG and GO analysis showed that there were no significant similarities in pathway enrichment between dexamethasone and 20β-DHB or between aldosterone and 20β-DHB (data not shown). Analysis of all genes differentially regulated by 20β-DHB demonstrated significant downregulation of oxidative phosphorylation and mitochondrial pathways (Figure 2E-F, Supplementary Table 2-3) by both GO and KEGG analysis, none of which were enriched by dexamethasone or aldosterone.

Having demonstrated that 20β-DHB activates both GR and MR in adipose tissue, male C57BL/6J mice (8 weeks of age) were administered 20β-DHB (20 μg/day) via subcutaneous mini-pump for 7 days with concurrent administration of vehicle, GR antagonist RU486 (mifepristone, 6 mg/kg/day) or MR antagonist spironolactone (20 mg/kg/day) in drinking water. We found that administration of 20β-DHB impaired glucose tolerance in wild-type mice and that this effect was ameliorated by antagonism of either GR or MR (Figure 2G-H). Fasting glucose was not different between the groups (Figure 2I). We selected four genes uniquely downregulated by 20β-DHB in subcutaneous adipose tissue and overrepresented in KEGG and GO analysis enrichment and determined their expression in these mice, we found both RU486 and spironolactone normalised expression (Supplementary Figure S1).
Figure 2: 20β-DHB induces both GR and MR regulated genes in adipose tissue. (A) mRNA expression of glucocorticoid receptor (GR), mineralocorticoid receptor (MR), GR-responsive genes Patatin Like Phospholipase Domain Containing 2 (Pnpla2) which encodes adipose triglyceride lipase (Atgl), Tsc22d3 which encodes Glucocorticoid-induced leucine zipper protein (GilZ), period 1 (Per1), tumour necrosis factor α (Tnfα) and MR-responsive gene Prostaglandin D2 synthase (Ptgs2) (n = 6/group). Data are mean ± SEM. Statistical significance was assessed by Mann-Whitney U test and two-way ANOVA as appropriate. *p < 0.05 relative to the wild-type control. (B) Venn diagram showing overlap of significantly differentially expressed genes (DEGs) in response to aldosterone, dexamethasone and 20β-DHB in subcutaneous adipose tissue. (C) Scatterplots of DEGs in response to 20β-DHB (red), aldosterone (blue) and both ligands (green). (D) Scatterplots of DEGs in response to 20β-DHB (red), dexamethasone (blue) and both ligands (green). (E) KEGG analysis of the 20β-DHB mediated transcriptome showing the log p-value for pathway enrichment. (F) Gene Ontology analysis of 20β-DHB mediated
transcriptome showing the log p-value for pathway enrichment. (G) Glucose tolerance tests (GTT) in male mice administered 20β-DHB with concurrent vehicle, RU486 or spironolactone (n= 4-5 mice/group) (H) Area under the curve for GTT in the four groups. (I) Fasting plasma glucose concentrations. Data are mean ± SEM. Statistical significance was assessed by ANOVA. *p < 0.05, **p < 0.01.

3.3 Male but not female Cbr1 haploinsufficient mice have reduced 20β-DHB in adipose tissue

In order to determine physiological effects of Cbr1/20β-DHB on glucose homeostasis in the lean and obese state, mice heterozygous for Cbr1 deletion were generated (Cbr1+/−). Male and female control (Cbr1+/+) and heterozygous (Cbr1+/−) littersmates were born at the expected Mendelian ratio but no homozygotes (Cbr1−/−) were born confirming that homozygosity of this gene deletion is foetal lethal. Cbr1 mRNA expression in subcutaneous adipose tissue of Cbr1−/− mice was approximately 20-30% of that in Cbr1+/+ control littersmates (Figure 3A). CBR1 protein expression in the subcutaneous adipose of male Cbr1−/− mice was approximately 50% of that of their control littersmates (Figure 3B-C). This model is globally deficient of Cbr1, which was confirmed by the gene expression and protein levels in liver and kidney (Supplementary Figure S2). Female mice had a similar reduction in mRNA, protein and activity (Figure 3A-D).

Analysis of the glucocorticoid profile by LC-MS/MS demonstrated that male Cbr1+/− mice had around a 50% less 20β-DHB in plasma and an 80% less in subcutaneous adipose tissue compared to Cbr1+/+ control littersmates (Figure 3E-F). Female mice had a similar reduction in plasma 20β-DHB concentrations but there was no difference in adipose tissue 20β-DHB content (Figure 3E-F), adipose corticosterone content was not different between genotypes (Supplementary Figure S3A). Plasma corticosterone and aldosterone concentrations were not different between genotypes (Figure 3G-H).

Figure 3: Lean Cbr1 heterozygous mice have reduced 20β-DHB in plasma and adipose tissue (A) Cbr1 mRNA expression in subcutaneous adipose tissue of lean male and female mice (n= 5-7 mice/group). (B) Representative Western blotting of CBR1 in subcutaneous adipose tissue from lean male Cbr1+/+ and Cbr1+/− mice (n= 4 mice/group). (C) Quantification of CBR1 in subcutaneous adipose tissue from lean male and female Cbr1+/+ and Cbr1+/− mice. (D) CBR1 activity in subcutaneous adipose tissue from lean male and female Cbr1+/+ and Cbr1+/− mice. (E-F) 20β-DHB quantified by LC-MS/MS in plasma and subcutaneous adipose tissue from lean male and female Cbr1+/+ and Cbr1+/− mice (n= 4-6 mice/group). (G-H) Plasma corticosterone and aldosterone quantified by LC-MS/MS in plasma from lean male and female Cbr1+/+ and Cbr1+/− mice (n= 5-6 mice/group). Data are expressed as mean ± SEM. Statistical analysis was conducted with the Mann–Whitney U test. *p < 0.05, **p < 0.01, and ***p < 0.001.
3.4 *Cbr1* haploinsufficiency improves glucose tolerance in lean male but not female mice but does not protect against the effects of high-fat feeding

Male *Cbr1*+/− mice had lower fasting glucose on a control chow diet and a smaller area under the curve for plasma glucose following intra-peritoneal glucose tolerance test indicating improved glucose tolerance compared to *Cbr1*+/+ mice. Fasting plasma insulin, insulin tolerance and fasting plasma non-esterified fatty acid (NEFA) did not differ between genotypes (Figure 4A-G). Female *Cbr1*+/− mice, however, showed no difference in fasting glucose or glucose tolerance compared to their littermate controls on a control chow diet (Supplementary Figure S4). When fed a control chow diet (4-8 weeks of age) there were no differences between littermates controls and *Cbr1*+/− in bodyweight, lean or fat mass, food or water intake between male (Figure 4H-K) or female (Supplementary Figure S4).

![Figure 4](image-url)

**Figure 4: Deletion of Cbr1 improves glucose tolerance in lean male mice.** (A) Glucose tolerance tests (GTT) in *Cbr1*+/+ and *Cbr1*+/− mice (n= 14 mice/group). (B) Area under the curve for GTT. (C-D) Fasting plasma glucose and insulin concentrations in *Cbr1*+/+ and *Cbr1*+/− mice (n= 6-10 mice/group). (E) Insulin tolerance test (ITT) in *Cbr1*+/+ and *Cbr1*+/− mice (n= 6 mice/group). (F) ITT area under curve. (G) Fasting plasma concentrations of non-esterified fatty acid (NEFA). (H-I) Lean mass and fat mass as percentage of bodyweight in *Cbr1*+/+ and *Cbr1*+/− mice (n= 4-6 mice/group). (J-K) Average food and water intake normalized to bodyweight in *Cbr1*+/+ and *Cbr1*+/− mice (n= 3-5 mice/group). Data are expressed as mean ± SEM. Statistical analysis was conducted with the Mann–Whitney U test. *p < 0.05, **p < 0.01, and ***p < 0.001.
We confirmed that these effects on metabolism were due to the role of Cbr1 in glucocorticoid metabolism by administering 20β-DHB in drinking water (100 μg/mL) for 7 days to male mice. Administration of 20β-DHB abolished the genotype difference in fasting glucose and glucose tolerance observed on a control chow diet (Figure 5).

Figure 5: Supplementation with 20β-DHB abolishes genotype differences in glucose tolerance. (A) Plasma 20β-DHB concentrations before and after supplementation in drinking water for 7 days in male Cbr1+/+ and Cbr1+-/- mice (n= 6-7 mice/group). (B) Glucose tolerance tests (GTT) prior to and following 7 days of supplementation with 20β-DHB (n= 8-9 mice/group). (C) Area under the curve for GTT. (D) Fasting plasma glucose prior to and following 7 days of supplementation with 20β-DHB (n=5-8 mice/group). Data are expressed as mean ± SEM. Statistical analysis was conducted with a Mann-Whitney U test and a 2-way ANOVA and post-hoc Bonferroni correction. ∗p < 0.05, ∗∗p < 0.01, and ∗∗∗p < 0.001.

We have previously shown that Cbr1 is increased in adipose tissue in mice on a high-fat diet. To determine the effect of Cbr1 deletion in obesity, male mice were fed a high-fat diet for 8 weeks. Transcript levels and plasma 20β-DHB concentrations were still reduced in Cbr1+-/- mice compared with littermate Cbr1+/+ controls but plasma corticosterone was not different (Figure 6A-C). Weight gain, lean mass, fat mass did not differ between the genotypes following high-fat feeding in males (Figure 6D) or females (Supplementary Figure S4). High-fat feeding of male mice abolished the difference between genotypes in fasting glucose and glucose tolerance; fasting insulin remained similar (Figure 6E-H). Female Cbr1+-/- mice continued to demonstrate no significant differences in metabolic parameters on a high-fat diet compared with littermate controls (Supplementary Figure S4).
3.5 Cbr1 overexpression in adipose tissue increases adipose 20β-DHB concentration

To test the hypothesis that adipose tissue Cbr1 specifically mediates the effects on systemic glucose tolerance we generated adipose-specific over-expressors of Cbr1 (R26-Cbr1Adpq) by crossing conditional knock-in mice with Adiponectin-Cre mice. mKate expression, used as a surrogate for recombination, was only detected in the adipose tissue of R26-Cbr1Adpq mice and not in R26-Cbr1Fl (data not shown). mRNA expression of Cbr1 was approximately 60% higher in R26-Cbr1Adpq male mice compared with floxed littermate controls but only 20% higher in female R26-Cbr1Adpq mice (Figure 7A). Protein and activity were approximately doubled in male and female R26-Cbr1Adpq mice compared with floxed controls (Figure 7B-C). R26-Cbr1Adpq had approximately twice as much 20β-DHB in their subcutaneous adipose tissue as floxed littermate controls (1.6 versus 4.2 ng/g adipose, p=0.0003) (Figure 7D-F). Adipose corticosterone content was not different between genotypes (Supplementary Figure S3B). There were no differences in plasma 20β-DHB or corticosterone concentrations (Figure 7G). There was no increase in mRNA expression in liver of R26-Cbr1Adpq mice compared with controls (Supplementary Figure S5).
Figure 7: Mice overexpressing Cbr1 in adipose tissue have increased 20β-DHB in adipose tissue but not in plasma. (A) Cbr1 mRNA expression in subcutaneous adipose tissue of male and female mice (n= 4-6 mice/group). (B) Representative Western blotting of CBR1 in subcutaneous adipose tissue from male R26-Cbr1F1 and R26-Cbr1Adpq mice (n= 4 mice/group). (C) Quantification of CBR1 in subcutaneous adipose tissue from male and female R26-Cbr1F1 and R26-Cbr1Adpq mice. (D) CBR1 activity in subcutaneous adipose tissue from male and female R26-Cbr1F1 and R26-Cbr1Adpq mice (n= 4 mice/group). (E-F) 20β-DHB quantified by LC-MS/MS in plasma and subcutaneous adipose tissue from male and female R26-Cbr1F1 and R26-Cbr1Adpq mice (n= 4-6 mice/group). (G) Plasma corticosterone quantified by LC-MS/MS in plasma from male and female R26-Cbr1F1 and R26-Cbr1Adpq mice (n= 4-5 mice/group). Data are expressed as mean ± SEM. Statistical analysis was conducted with the Mann–Whitney U test and 2-way ANOVA. *p < 0.05, **p < 0.01.

3.6 Adipose-specific overexpression of Cbr1 worsens metabolic status in lean mice but does not exacerbate the effects of high-fat feeding

When fed a control chow diet there were no differences in bodyweight, lean or fat mass, between male or female R26-Cbr1Adpq and their floxed littermate controls (Supplementary Figure S6). Male R26-Cbr1Adpq mice had higher fasting glucose on a chow diet (9.5 ± 0.3 v 8.4 ± 0.3, p=0.04) and a larger area under the curve of plasma glucose following intra-peritoneal glucose tolerance test (1819 ± 66 v 1392 ± 14, p=0.03) (Figure 8A-C). Female R26-Cbr1Adpq mice had a larger area under the curve of plasma glucose following intra-peritoneal glucose tolerance test but fasting glucose was not different between the genotypes (Figure 8D-F). Fasting insulin and fasting NEFA did not differ between the genotypes in either sex on either diet (Supplementary Figure S6). High-fat feeding of male and female mice abolished the difference between genotypes in fasting glucose and glucose tolerance (Figure 8A-F). mRNA expression of GR and MR responsive genes in subcutaneous adipose tissue showed a similar pattern to that seen when 20β-DHB was administered to adrenalectomised mice (Figure 8G; Figure 2A). GR and MR levels were unaltered, GR-induced genes Pnpla2, Tsc22d3 and Per1 were increased in R26-Cbr1Adpq. Tnfa, which is downregulated by GR activation, was unaltered by genotype (Figure 8G). Expression of MR-responsive gene Ptgds was also increased in R26-Cbr1Adpq compared with floxed controls (Figure 8G). These results are consistent with a mixed GR and MR activation in adipose tissue.
Figure 8: Adipose-specific overexpression of Cbr1 worsens metabolic status on a chow diet but does not exacerbate the effects of high-fat feeding. (A) Glucose tolerance tests (GTT) in male R26-Cbr1F1 and R26-Cbr1Adpq mice on a chow diet and after high-fat feeding (n= 5-7 mice/group). (B) Area under the curve for GTT in male mice. (C) Fasting plasma glucose concentrations in male mice R26-Cbr1F1 and R26-Cbr1Adpq (n= 6-10 mice/group). (D) Glucose tolerance tests (GTT) in female mice R26-Cbr1F1 and R26-Cbr1Adpq on a chow diet and after high-fat feeding (n= 6-9 mice/group). (E) Area under the curve for GTT in female mice. (F) Fasting plasma glucose concentrations in female mice R26-Cbr1F1 and R26-Cbr1Adpq (n= 6-8 mice/group). (G) mRNA expression of glucocorticoid receptor (GR), mineralocorticoid receptor (MR), GR-responsive genes Patatin Like Phospholipase Domain Containing 2 (Pnpla2) which encodes adipose triglyceride lipase (Atgl), Tsc22d3 which encodes Glucocorticoid-induced leucine zipper protein (Gilz), period 1 (Per1), tumour necrosis factor α (Tnfα) and MR-responsive gene Prostaglandin D2 synthase (Ptdgs) in subcutaneous adipose tissue of male R26-Cbr1F1 and R26-Cbr1Adpq mice on a chow diet (n= 6-7/group). Data are mean ± SEM. Statistical significance was assessed by Mann-Whitney U test and ANOVA. *p < 0.05, **p < 0.01.
4. Discussion

In this study we have shown that carbonyl reductase 1 is a novel regulator of glucocorticoid and mineralocorticoid receptor activation in adipose tissue with a role in regulation of glucose homeostasis. We showed that 20β-DHB activates both GR and MR in adipose tissue but has a transcriptional profile which is distinct from either dexamethasone or aldosterone, characterised by downregulation of oxidative phosphorylation and mitochondrial pathways. Furthermore, we demonstrated that systemic administration of 20β-DHB worsens glucose tolerance and this effect is ameliorated by antagonism of both GR and MR. We found that Cbr1 haploinsufficiency improves glucose tolerance and lowers fasting glucose, but only when accompanied by a concurrent reduction in adipose as well as plasma 20β-DHB concentrations as seen in male but not female mice. Restoring 20β-DHB levels, without altering corticosterone levels, ‘rescued’ the phenotype of Cbr1+/− mice. The importance of adipose CBR1/20β-DHB in mediating the metabolic effects of Cbr1 was further supported by the phenotype observed in mice with adipose-specific overexpression of Cbr1, with both males and females demonstrating increased levels of 20β-DHB in adipose but not plasma and both sexes having worsened glucose tolerance. Finally, unlike other models of altered glucocorticoid action in adipose tissue, the influence of adipose CBR1/20β-DHB on glucose tolerance was not associated with altered fat mass or bodyweight and was attenuated by high fat feeding, suggesting that 20β-DHB modulates a different balance of effects compared with ‘conventional’ GR ligands.

Sex-specific effects of Cbr1 manipulation have been reported previously in studies investigating the role of Cbr1 in doxorubicin metabolism. Freeland et al observed that female Cbr1+/− mice were not protected against the cardiotoxic effects of doxorubicin to the same extent as male Cbr1+/− mice and suggested this was because tissue Cbr1 levels were higher in wild-type females than males, although they did not report enzyme activity [34]. Differences in CBRI expression in humans are thought to account for the increased susceptibility of women [35] and ethnic minorities to doxorubicin toxicity [36]. In our study, Cbr1 mRNA expression and activity were higher in adipose tissue in control females than males but haploinsufficiency resulted in similar low levels of mRNA expression and activity in males and females and is therefore unlikely to account for the differences between sexes. Explanations for the disparity between adipose Cbr1 and 20β-DHB levels in females include the following possibilities; CBRI is not the only enzyme responsible for 20β-DHB production in females; that removal of 20β-DHB from the adipose differs between sexes; or that in female adipose CBRI always favours corticosterone as a substrate whereas in males competitive or alternative substrates are available which are preferentially metabolised when CBRI levels are reduced. Female Cbr1+/− mice had a reduction in plasma concentrations of 20β-DHB but did not have a reduction in adipose tissue concentrations, suggesting that other tissues, such as the gut, may contribute more 20β-DHB to the circulating pool than adipose, supported by the finding that plasma levels were not increased in the adipose-specific Cbr1 overexpressing mice. The fact that adipose 20β-DHB and glucose tolerance was unaltered in female Cbr1+/− supports the hypothesis that adipose 20β-DHB is the driver of the phenotype observed in males. This is further supported by the presence of both increased 20β-DHB levels in adipose tissue of female R26-Cbr1Adpq and worsened glucose tolerance.

It appears from this study that CBRI/20β-DHB modulates systemic glucose tolerance via a paracrine effect in adipose tissue and not by endocrine signalling from adipose to other tissues. A reduction in plasma 20β-DHB, when not accompanied by reduction in adipose 20β-DHB, as in female Cbr1+/− mice, was not associated with an improvement in glucose tolerance. Conversely, in R26-Cbr1Adpq mice worsened glucose tolerance was present without a change in plasma 20β-DHB. Administering 20β-DHB systemically resulted in plasma concentrations of more than ten times physiological concentrations but the change in glucose tolerance was of a similar magnitude to that seen with only a doubling of adipose 20β-DHB concentration in R26-Cbr1Adpq. It is well documented that manipulation of glucocorticoid action in adipose tissue can have systemic effects on metabolic parameters, for example mice over-expressing 11β-HSD1 in adipose tissue have glucose intolerance but unlike Cbr1
over-expressors they also demonstrate increased fat mass and free fatty acids [3]. The difference in phenotype between these two models, both of which increase corticosteroid receptor activation, is most likely due to ligand-specific transcriptional responses when receptors are activated by 20β-DHB. It may also be due to a particular balance of GR and MR activation induced by 20β-DHB, in which our data suggest relatively potent MR activation; the interaction of GR and MR in determining metabolic parameters is complex and still incompletely understood with conflicting data from genetic models manipulating each receptor [1; 2; 7; 37; 38].

Glucocorticoids acting on GR and MR have diverse effects on adipose tissue biology including reducing glucose uptake, increasing lipolysis, regulating inflammation and adipokine release. Our study shows that 20β-DHB is a ligand of both receptors and though our in vitro data suggested that there would be a preferential activation of MR, the in vivo investigations made it apparent that the most pronounced functional effects on glucose tolerance are ameliorated by antagonism of both GR and MR. Impairment of glucose tolerance by decreasing glucose uptake and metabolism is induced by excess dexamethasone activating GR, excess corticosterone activating both GR and MR and overexpression of MR in adipose tissue [4-7]. It has also been shown that combined antagonism of GR and MR improves glucose tolerance [39; 40]; the effect of 20β-DHB demonstrated here is therefore consistent with that of a mixed GR/MR agonist. Interestingly, the effects of dexamethasone on glucose uptake are more marked under basal conditions compared with insulin-stimulated conditions which may also be the case for 20β-DHB given that overexpression of Cbr1 did not worsen glucose tolerance during high-fat feeding [41]. We found no evidence for an effect of CBR1/20β-DHB on lipolysis or inflammatory markers within the adipose tissue. Tnfα expression was not downregulated by 20β-DHB and there were no inflammatory pathways significantly downregulated by 20β-DHB. That 20β-DHB has divergent effects compared with other glucocorticoids and aldosterone is unsurprising given that these receptors induce ligand-specific effects [42]. The distinct nature of the transcriptional response is, in part, due to co-regulator recruitment [43] and though 20β-DHF, on binding to MR, recruited almost 93% of the co-regulators recruited by the binding of aldosterone or cortisol it is clear that even a very small differences in co-regulator recruitment can result in a marked difference in transcriptional response [44]. Moreover, the assay that we used only probes co-regulator interactions with the receptor ligand binding domain, and not the N-terminal part of the receptor. Our transcriptomic analysis demonstrated that whilst a significant number of 20β-DHB associated DEGs were shared with dexamethasone (54%), only 15% were shared with aldosterone and that there was no commonality in pathway enrichment between the ligands. We would suggest therefore, that 20β-DHB induces a ligand-specific response when binding adipose GR and MR due to the large (GR) or subtle (MR) differences in co-regulator recruitment [45] or potentially the formation of heterodimers of the receptors which was not investigated in this study [46].

That Cbr1 deletion did not protect from the effects of a high-fat diet is somewhat surprising, particularly given the increase in Cbr1 observed in obese adipose in humans and mice [15]. One explanation for this is the role of CBR1 in the context of oxidative stress [47]. CBR1 is upregulated in oxidative stress; it inactivates highly reactive lipid-aldehydes [47], ameliorates lipid peroxidation [48] and when overexpressed in hepatic cell lines confers protection against reactive oxygen species induced cell damage [49]. CBR1 in pancreatic β cells appears to attenuate apoptosis and increase cell survival and insulin secretion in vitro under glucotoxic conditions [50]. It may be, therefore, that under normal diet conditions a lack of CBR1 is beneficial due to a reduction in GR/MR activation by 20β-DHB but when oxidative stress increases in obesity its absence is detrimental thus cancelling out any protective effect. Equally, whilst overexpression in the adipose of lean mice may be detrimental due to chronic activation of GR/MR, it is likely to be beneficial when adipose expands and there is an increase in reactive oxygen species. Alternative endogenous substrates of CBR1 include prostaglandins [47; 51], indeed the foetal lethality of Cbr1−/− mice has been attributed to altered
prostaglandin degradation in the amnion or uterus [22]. High-fat feeding is associated with inflammation and increase in prostaglandins in adipose tissue [52]. Reduction in CBR1 may result in excess prostaglandin content within the adipose tissue which mitigates the protective effect of reduced GR/MR activation.

In conclusion, we have, for the first time, described CBR1/20β-DHB as a novel mediator of glucocorticoid action in adipose tissue with a significant impact on systemic glucose homeostasis in the lean state. There is a very wide variation in CBR1 expression in human populations [53-55]; common environmental factors such as cigarette smoke up-regulate the enzyme [56] and CBR1 inhibitors are present in many foods and supplements [57]. Our data suggest that these variations may have important consequences for an individual’s glucocorticoid metabolism and metabolic health and that these consequences should be considered when manipulating CBR1 for other reasons such as in cancer treatment.
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Supplementary Methods

S1.1 Quantification of steroids in plasma by LC-MS/MS

The mass spectrometer was operated at 600ºC with polarity switching in multiple reaction mode in negative mode (-4.5 kV) for Aldosterone at m/z 359.0 → 188.9 at -24V, 359.0 → 331.1 at -22V and d8Aldo at m/z 367.1 → 193.9 at -26V and in positive mode (5.5 kV) for B, 20β-DHB at m/z 347.1 → 121.1 at 29V, 347.1 → 90.0 at 75V, 365.2 → 269.1 at 25V and 365.2 → 121.0 at 33V and m/z 355.3 → 125.1 at 31V for d8-B. They eluted at 2.3, 2.3, 2.5, 3.4 and 3.3 mins.

S1.2 Quantification of steroids in adipose by LC-MS/MS

The mass spectrometer was operated in positive ion multiple reaction mode for 20β-DHB and d8-B at 349.1 → 91.1 at 71 V and 349.1 → 121.1 at 33 V and 355.3 → 125.1 at 31V, respectively. They eluted at 2.5 and 3.3 mins.

S1.3 RNA sequencing

Total RNA samples were quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc) and the Qubit RNA HS assay kit. RNA integrity was assessed using the Agilent 2100 Bioanlyser System (Agilent Technologies Inc) and Agilent RNA 6000 Nano kit. Libraries were prepared from 500ng of each total-RNA sample using the TruSeq Stranded mRNA Library Kit (Illumina Inc) according to the provided protocol. Poly-A containing mRNA molecules were purified using poly-T oligo attached magnetic beads. Following purification the mRNA was fragmented using divalent cations under elevated temperature and primed with random hexamers. Primed RNA fragments were reverse transcribed into first strand cDNA using reverse transcriptase and random primers. RNA templates were removed and a replacement strand synthesised incorporating dUTP in place of dTTP to generate ds cDNA. The incorporation of dUTP in second strand synthesis quenches the second strand during amplification as the polymerase used in the assay is not incorporated past this nucleotide. AMPure XP beads (Beckman Coulter) were then used to separate the ds cDNA from the second strand reaction mix, providing blunt-ended cDNA. A single ‘A’ nucleotide was added to the 3’ ends of the blunt fragments to prevent them from ligating to another during the subsequent adapter ligation reaction, and a corresponding single ‘T’ nucleotide on the 3’ end of the adapter provided a complementary overhang for ligating the adapter to the fragment. Multiple indexing adapters were then ligated to the ends of the ds cDNA to prepare them for hybridisation onto a flow cell, before 12 cycles of PCR were used to selectively enrich those DNA fragments that had adapter molecules on both ends and amplify the amount of DNA in the library suitable for sequencing. After amplification libraries were purified using AMPure XP beads. Libraries were quantified by fluorometry using the Qubit dsDNA HS assay and assessed for quality and fragment size using the Agilent Bioanlyser with the DNA HS Kit. Sequencing was performed using the NextSeq 500/550 High-Output v2 (150 cycle) Kit on the NextSeq 550 platform (Illumina Inc). 48 libraries were combined in three equimolar pools of 16 based on the library quantification results and each pool was run across a single High-Output Flow Cell.

The quality of the raw sequencing data was verified using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were aligned to the mouse genome (GRCm38) using STAR (https://academic.oup.com/bioinformatics/article/29/1/15/272537) and gene counts quantified based on the GENCODE M18 annotation.

The data were normalised using TMM (Trimmed Mean of M-values) (https://genomebiology.biomedcentral.com/articles/10.1186/gb-2010-11-3-r25) and transformed with voom (https://genomebiology.biomedcentral.com/articles/10.1186/gb-2014-15-2-r29), resulting in log2-counts per million (log-CPM) values with associated precision weights. Outlier detection of samples was performed using a combination of objective scoring methods: Hoeffding's D-statistic,
mean Pearson correlation with other samples, sum of Euclidean distance to other samples, and the Kolmogorov-Smirnov test statistic. No quality issues were identified within the data. Statistical analysis was subsequently performed using empirical Bayes from the limma R package (https://academic.oup.com/nar/article/43/7/e47/2414268) using the normalised log-CPM values with associated precision weights from voom. The statistical models evaluated each treatment relative to the DMSO control. Genes were considered significantly differentially expressed at p < 0.01 without adjustment for multiple testing. Following identification of putatively differentially-expressed genes, the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways [1] and Gene Ontology (GO) terms [2] were assessed for pathway enrichment using a hypergeometric test. Up- and down-regulated genes were evaluated separately and the GO terms analysis was performed across all three GO ontologies. KEGG pathways and GO terms were considered significant at p < 0.01 from hypergeometric testing.

[1] Kanehisa, M., Goto, S., 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Research 28(1):27-30.
[2] Ashburner, M., Ball, C.A., Blake, J.A., Butler, H., Cherry, J.M., Eppig, J.T., et al., 2001. Creating the Gene Ontology resource: Design and implementation. Genome Research 11(8):1425-1433.
Figure S1: mRNA transcript levels of selected 20β-DHB differentially regulated genes in subcutaneous adipose in mice treated with 20β-DHB with GR antagonist RU486 or MR antagonist spironolactone. * P<0.05 compared with vehicle. 20β-DHB downregulates genes encoding ATP synthase subunits 5a1, 5b, 5h and 5k. Treatment with RU486 and spironolactone normalised expression of these genes relative to the vehicle control.
Figure S2: Male Cbr1 heterozygous mice have reduced in liver and renal tissue. (A) Cbr1 mRNA expression in hepatic tissue of male mice (n= 5 mice/group). (B) Representative Western blotting of CBR1 in liver from male Cbr1+/+ and Cbr1+/− mice (n= 4 mice/group). (C) Quantification of CBR1 in liver from male Cbr1+/+ and Cbr1+/− mice. (D) Cbr1 mRNA expression in renal tissue of male mice (n=4 mice/group). (E) Representative Western blotting of CBR1 in kidney from male Cbr1+/+ and Cbr1+/− mice (n= 4 mice/group). (F) Quantification of CBR1 in kidney from male Cbr1+/+ and Cbr1+/− mice. Data are expressed as mean ± SEM. The statistical analysis was conducted with the Mann–Whitney U test. *p < 0.05, **p < 0.01, and ***p < 0.001.
Figure S3: Adipose corticosterone content was not different between the genotypes in either transgenic line.
Figure S4: Deletion of Cbr1 does not alter glucose tolerance in female mice. (A) Glucose tolerance tests (GTT) in Cbr1+/+ and Cbr1+/- mice (n= 5 mice/group) on chow or high-fat diet. (B) Area under the curve for GTT. (C-D) Fasting plasma glucose and insulin concentrations in Cbr1+/+ and Cbr1+/- mice on chow or high-fat diet (n= 5 mice/group). (E) Bodyweight gain with high-fat feeding in Cbr1+/+ and Cbr1+/- mice. (F) Lean mass and fat mass as percentage of bodyweight in Cbr1+/+ and Cbr1+/- mice on a chow diet measured by TD-NMR (n= 5 mice/group). (G) Lean mass and fat mass as percentage of bodyweight in Cbr1+/+ and Cbr1+/- mice on a high-fat diet measured by TD-NMR (n= 5 mice/group). Data are expressed as mean ± SEM. The statistical analysis was conducted with the Mann–Whitney U test. *p < 0.05, **p < 0.01, and ***p < 0.001.
**Figure S5**

Hepatic *Cbr1* mRNA expression was not different in male and female *R26-Cbr1<sup>F1</sup>* mice compared with controls.
Figure S6

Figure S6: Adipose-specific overexpression of Cbr1 does not affect bodyweight, insulin or NEFAs on a chow diet in males or females. (A) Bodyweight over time in male R26-Cbr1Fl and R26-Cbr1Adpq mice on a chow diet and after high-fat feeding (n= 5-7 mice/group). (B) Lean and fat mass percentage in male R26-Cbr1Fl and R26-Cbr1Adpq mice on a chow diet. (C) Fasting plasma insulin concentrations in male mice R26-Cbr1Fl and R26-Cbr1Adpq (n= 5 mice/group). (D) Fasting plasma non-esterified fatty acid concentrations in male mice R26-Cbr1Fl and R26-Cbr1Adpq (n= 4 mice/group). (E) Bodyweight over time in female R26-Cbr1Fl and R26-Cbr1Adpq mice on a chow diet and after high-fat feeding (n= 5-7 mice/group). (F) Lean and fat mass percentage in female R26-Cbr1Fl and R26-Cbr1Adpq mice on a chow diet. (G) Fasting plasma insulin concentrations in female mice R26-Cbr1Fl and R26-Cbr1Adpq (n= 5 mice/group). (H) Fasting plasma non-esterified fatty acid concentrations in female mice R26-Cbr1Fl and R26-Cbr1Adpq (n= 4 mice/group). Data are mean ± SEM. Statistical significance was assessed by Mann-Whitney U test and ANOVA* indicates significance at p < 0.05, **p < 0.01.
## Supplementary Tables

### Supplementary Table 1 Primer details for qPCR

| Gene Symbol, full name | Forward Primer (3’→ 5’) | Reverse Primer (5’→ 3’) |
|------------------------|--------------------------|-------------------------|
| RNA18s, ribosomal RNA 18s | CTCAACACGGGAACCTCAC | CGCTCCACAAACTAAGAAGC |
| Tbp, TATA-binding protein | GGGAGAATCATGGACCAGAA | GATGGGAATTCCAGGAGTCA |
| β-actin | CTAAGGCCAACCGTGAAAG | ACCAGAGGCATACAGGGACA |
| Chr1, Carbonyl Reductase 1 | AGGTGACAATGAAAACGAAC | GGAACATCCACACCTCTCTG |
| mKate | GCACCCAGACCATGAATCAAG | CTGCCGTACATGAAGCTGGTA |
| GR, Glucocorticoid receptor (Nr3c1) | CAAAAGATTGCAGGTATCCTATGAA | CTTGGCTCTTCAGACCTTCC |
| MR, Mineralocorticoid receptor (Nr3c2) | CAAAAGAGGCCGTGAAGGG | TTTCTCCGAATCTTATCAATAATGC |
| Pnpla2, Adipose triglyceride lipase (Atgl) | GTTCCTTTTGTTCCACACAG | CCTCTGAAGGCCTCTCTCTT |
| Tsc22d3, Glucocorticoid-induced leucine zipper protein (Gilt) | AGGTGGTCTTCCAGGAGGTC | TCCGTTAAGCTGGAATACAGTGC |
| Per1, Period | GCTTCGTTGGACTGGACACCT | TGGTTTAGATCGGCAGTGG |
| Tnfa, Tumour necrosis factor alpha | TGAGGAAAGCTGGCATTG | GGCGCTCTACCTTCAGACC |
| Ptdgs, prostaglandin D2 synthase | CTTCACAGGACGAAGGTTCC | CGGGTTCACACTGGGTTT |

**Supplementary Table 2 Gene Ontology (GO) terms enriched in adipose exposed to 20β-dihydrocorticosterone compared with DMSO**

| GO Term                        | Genes                                                                 | Number of significant genes | Number of genes in term | % of genes identified as significant within the term | Odds ratio-number of significant genes over that expected | Raw (unadjusted) p-value from hypergeometric test | Adjusted p-value [Benjamini and Hochberg 1995] |
|-------------------------------|----------------------------------------------------------------------|-----------------------------|-------------------------|-----------------------------------------------------|--------------------------------------------------------|---------------------------------------------------|-----------------------------------------------|
| mitochondrion                 | 170002F05Rik, 2010107E04Rik, 2410015M20Rik, Abcb8, Acaca2, Acads, Acadvl, Acly, Ac02, Acfs2, Acfs3, Acsl3, Acsl5, Adcy10, Akr1b10, Atpsa1, Atpsb5, Atpsd5, Atps5e, Atps5g2, Atps5h, Atps5j2, Atps5k, Atppa2, Bok, Cars2, Cdc51, Cdc58, Chchd10, Cipap1, Cidea, Cisd3, Cmcc2, Coasy, Coq6, Coq7, Cox10, Coxl6, Cox4I1, Cox4I2, Cox5a, Cox6b1, Cox7a1, Cpx7c, Cox8b, Cym, Cysc1, Cysc, Dlat, Dist, Dnaq11, Ectf, Etfb, Gars, Gpd1, Gpd2, Grpe1, Hadha, Hccs, Hdc2, Hdh3, Higaa1, Hk2, Kirj8, Lace1, Lacmt2, Lats2, Ldha, Ldhb, Letn1, Letmd1, Lipt1, Lrkr1, Mdh2, Minos1, Mpc1, Mpc2, Mrpl12, Mrpl16, Mrpl30, Mrpl34, Mrpl37, Mrpl51, Mrps10, Mrps27, Mrps28, Mrps35, Mtfj1, Mthfd2, Ndufa1, Ndufa11, Ndufa5, Ndufa9, Ndufb11, Ndufb5, Ndufb7, Ndufb8, Ndufs4, Ndufs5, Ndufs6, Ndufs8, Ndufv1, Nipsnap1, Pdhb, Pdp2, Pfdn4, Phb, Pkm, Prka, Prpl, Prkaca, Ptc2, Raf1, Rhot2, Rilp, Rmdn3, Samm50, Sars2, Sdhb, Sdha, Sept4, Sirt5, Slc25a19, Slc25a35, Sod2, Sax4, Sulp1, Tors2, Tbra4, Timm17a, Timm44, Timm50, Tmem134c, Tmem12d, Ucp3, Uqcr10, Uqcr11, Uqcr1, Uqcrh, Vars2, Vdac2, Xrcc3, Zadh2 | 146 | 1701 | 8.58 | 6.20 | 3.146e-52 | 9.012e-49 |
|                  | 2410015M20Rik, Abcb8, Acaca2, Acads, Acadvl, Acly, Ac02, Acfs2, Acfs3, Acsl3, Acsl5, Atpsa1, Atpsb5, Atpsd5, Atps5e, Atps5g2, Atps5h, Atps5j2, Atps5k, Atppa2, Bok, Cars2, Cdc51, Cdc58, Chchd10, Cipap1, Cidea, Cisd3, Cmcc2, Coasy, Coq6, Coq7, Cox10, Coxl6, Cox4I1, Cox4I2, Cox5a, Cox6b1, Cpx7c, Cox8b, Cym, Cysc1, Cysc, Dlat, Dist, Dnaq11, Ectf, Etfb, Gars, Gpd1, Gpd2, Grpe1, Hadha, Hccs, Hdc2, Hdh3, Higaa1, Hk2, Kirj8, Lace1, Lacmt2, Lats2, Ldha, Ldhb, Letn1, Letmd1, Lipt1, Lrkr1, Mdh2, Minos1, Mpc1, Mpc2, Mrpl12, Mrpl16, Mrpl30, Mrpl34, Mrpl37, Mrpl51, Mrps10, Mrps27, Mrps28, Mrps35, Mtfj1, Mthfd2, Ndufa1, Ndufa11, Ndufa5, Ndufa9, Ndufb11, Ndufb5, Ndufb7, Ndufb8, Ndufs4, Ndufs5, Ndufs6, Ndufs8, Ndufv1, Nipsnap1, Pdhb, Pdp2, Pfdn4, Phb, Pkm, Prka, Prpl, Prkaca, Ptc2, Raf1, Rhot2, Rilp, Rmdn3, Samm50, Sars2, Sdhb, Sdha, Sept4, Sirt5, Slc25a19, Slc25a35, Sod2, Sax4, Sulp1, Tors2, Tbra4, Timm17a, Timm44, Timm50, Tmem134c, Tmem12d, Ucp3, Uqcr10, Uqcr11, Uqcr1, Uqcrh, Vars2, Vdac2, Xrcc3, Zadh2 | 97 | 739 | 13.13 | 8.80 | 3.155e-49 | 4.520e-46 |
| mitochondrial part             | 2410015M20Rik, Abcb8, Acaca2, Acads, Acadvl, Acly, Ac02, Acfs2, Acfs3, Acsl3, Acsl5, Atpsa1, Atpsb5, Atpsd5, Atps5e, Atps5g2, Atps5h, Atps5j2, Atps5k, Atppa2, Bok, Cars2, Cdc51, Cdc58, Chchd10, Cipap1, Cidea, Coasy, Coq6, Coq7, Cox10, Coxl6, Cox4I1, Cox4I2, Cox5a, Cox6b1, Cpx7c, Cox8b, Cs, Cysc1, Cysc, Dlat, Dnaq11, Ectf, Etfb, Gpd2, Grpe1, Hadha, Hccs, Higaa1, Hk2, Lacmt2, Letn1, Letmd1, Mdh2, Minos1, Mpc1, Mpc2, Mrpl12, Mrpl16, Mrpl30, Mrpl34, Mrpl37, Mrpl51, Mrps10, Mrps27, Mrps28, Mrps35, Mtfj1, Mthfd2, Ndufa1, Ndufa11, Ndufa5, Ndufa9, Ndufb11, Ndufb5, Ndufb7, Ndufb8, Ndufs4, Ndufs5, Ndufs6, Ndufs8, Ndufv1, Nipsnap1, Pdhb, Pdp2, Pfdn4, Phb, Pkm, Prka, Prpl, Prkaca, Ptc2, Raf1, Rhot2, Rilp, Rmdn3, Samm50, Sars2, Sdhb, Sdha, Sept4, Sirt5, Slc25a19, Slc25a35, Sod2, Sax4, Sulp1, Tors2, Tbra4, Timm17a, Timm44, Timm50, Tmem134c, Tmem12d, Ucp3, Uqcr10, Uqcr11, Uqcr1, Uqcrh, Vars2, Vdac2, Xrcc3, Zadh2 | 73 | 398 | 18.34 | 12.33 | 6.128e-47 | 5.853e-44 |
|                  | 2410015M20Rik, Abcb8, Acaca2, Acads, Acadvl, Acly, Ac02, Acfs2, Acfs3, Acsl3, Acsl5, Atpsa1, Atpsb5, Atpsd5, Atps5e, Atps5g2, Atps5h, Atps5j2, Atps5k, Atppa2, Bok, Cars2, Cdc51, Cdc58, Chchd10, Cipap1, Cidea, Coasy, Coq6, Coq7, Cox10, Coxl6, Cox4I1, Cox4I2, Cox5a, Cox6b1, Cpx7c, Cox8b, Cs, Cysc1, Cysc, Dlat, Dnaq11, Ectf, Etfb, Gpd2, Grpe1, Hadha, Hccs, Higaa1, Letn1, Letmd1, Mdh2, Minos1, Mpc1, Mpc2, Mtfj1, Ndufa1, Ndufa11, Ndufa5, Ndufa9, Ndufb11, Ndufb5, Ndufb7, Ndufb8, Ndufs4, Ndufs5, Ndufs6, Ndufs8, Ndufv1, Nipsnap1, Phb, Ppif, Rho2, Rmdn3, Samm50, Sars2, Sdhb, Sdha, Sept4, Sirt5, Slc25a19, Slc25a35, Sod2, Sax4, Sulp1, Tors2, Tbra4, Timm17a, Timm44, Timm50, Tmem134c, Ucp3, Uqcr10, Uqcr11, Uqcr1, Uqcrh, Vars2, Vdac2, Xrcc3, Zadh2 | 74 | 438 | 16.89 | 11.17 | 5.818e-45 | 4.167e-42 |
| mitochondrial envelope | Ndufs6, Ndufs8, Ndufv1, Nipsnap1, P2rx5, Phb, Ppif, Rhot2, Samm50, Sdhb, Sdhc, Sirt5, Slc25a19, Slc25a35, Sod2, Suclg1, Timm17a, Timm44, Timm50, Timem14c, Ucp3, Uqcr10, Uqcr11, Uqcr1, Uqcrh, Vdac2 | 2410015M20Rik, Abcb8, Acaa2, Acads, Acadvl, Acsl3, Acsl5, Atp5a1, Atp5b, Atp5d, Atp5e, Atp5g2, Atp5h, Atp5j2, Atp5k, Atp5o, Bok, Chchd10, Cidea, Coq6, Coq7, Cox10, Cox16, Cox4i1, Cox4i2, Cox5a, Cox6b1, Cox7a1, Cox7a2, Cox7c, Cox8b, Cyc1, Cyc6, Dnaq11, Gpd2, Grep1, Hadha, Hccs, Hips1a, Hk2, Letm1, Letm2, Mino1, Mpc1, Mpc2, Mtfp1, Ndufa1, Ndufa11, Ndufa5, Ndufa9, Ndufb11, Ndufb5, Ndufb7, Ndufb8, Ndufs4, Ndufs5, Ndufs6, Ndufs8, Ndufv1, Nipsnap1, Phb, Ppif, Rhot2, Rmdn3, Samm50, Sdhb, Sdhc, Sirt5, Slc25a19, Slc25a35, Sod2, Suclg1, Timm17a, Timm44, Timm50, Timem14c, Ucp3, Uqcr10, Uqcr11, Uqcr1, Uqcrh, Vdac2 | 83 | 586 | 14.16 | 9.25 | 2.066e-44 | 1.184e-41 |
Supplementary Table 3 Kyoto Encyclopedia of Genes and Genomes (KEGG) terms enriched in adipose exposed to 20β-dihydrocorticosterone compared with DMSO

| KEGG Term                        | Genes                                                                 | Number of significant genes | Number of genes in term | % of genes identified as significant within the term | Odds ratio-number of significant genes over that expected | Raw (unadjusted) p-value from hypergeometric test | Adjusted p-value (Benjamini and Hochberg 1995) |
|----------------------------------|-----------------------------------------------------------------------|----------------------------|-------------------------|-----------------------------------------------------|---------------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Oxidative phosphorylation       | Atp4b, Atp5a1, Atp5b, Atp5d, Atp5e, Atp5g2, Atp5h, Atp5o, Cox10, Cox4i1, Cox4i2, Cox5a, Cox6b1, Cox7a1, Cox7a2, Cox7c, Cox8b, Cyc1, Ndufa1, Ndufa11, Ndufa5, Ndufa9, Ndufb11, Ndufb5, Ndufb7, Ndufb8, Ndufs4, Ndufs5, Ndufs6, Ndufs8, Ndufv1, Sdhb, Sdhd, Uqcr10, Uqcr11, Uqcr12, Uqcr13 | 39                          | 133                      | 29.32                                               | 18.32                                                   | 1.642e-30                                      | 2.775e-28                                      |
| Parkinson's disease              | Atp5a1, Atp5b, Atp5d, Atp5e, Atp5g2, Atp5h, Atp5o, Cox4i1, Cox4i2, Cox5a, Cox6b1, Cox7a1, Cox7a2, Cox7c, Cox8b, Cyc1, Cycs, Ndufa1, Ndufa11, Ndufa5, Ndufa9, Ndufb11, Ndufb5, Ndufb7, Ndufb8, Ndufs4, Ndufs5, Ndufs6, Ndufs8, Ndufv1, Ppif, Prkaca, Sdhb, Sdhd, Uqcr10, Uqcr11, Uqcr12, Uqcr13, Uqcr14, Vdac2 | 39                          | 142                      | 27.46                                               | 16.70                                                   | 2.686e-29                                      | 2.269e-27                                      |
| Huntington's disease             | Atp5a1, Atp5b, Atp5d, Atp5e, Atp5g2, Atp5h, Atp5o, Cox4i1, Cox4i2, Cox5a, Cox6b1, Cox7a1, Cox7a2, Cox7c, Cox8b, Cyc1, Cycs, Dnah7a, Ndufa1, Ndufa11, Ndufa5, Ndufa9, Ndufb11, Ndufb5, Ndufb7, Ndufb8, Ndufs4, Ndufs5, Ndufs6, Ndufs8, Ndufv1, Polr2g, Ppif, Sdhb, Sdhd, Sod2, Uqcr10, Uqcr11, Uqcr12, Uqcr13, Uqcr14, Uqcr15, Vdac2 | 41                          | 191                      | 21.47                                               | 12.13                                                   | 3.429e-26                                      | 1.932e-24                                      |
| Alzheimer's disease              | Atp5a1, Atp5b, Atp5d, Atp5e, Atp5g2, Atp5h, Atp5o, Calm5, Cox4i1, Cox4i2, Cox5a, Cox6b1, Cox7a1, Cox7a2, Cox7c, Cox8b, Cyc1, Cycs, Ndufa1, Ndufa11, Ndufa5, Ndufa9, Ndufb11, Ndufb5, Ndufb7, Ndufb8, Ndufs4, Ndufs5, Ndufs6, Ndufs8, Ndufv1, Sdhb, Sdhd, Uqcr10, Uqcr11, Uqcr12, Uqcr13, Uqcr14, Uqcr15, Uqcr16 | 37                          | 174                      | 21.26                                               | 11.69                                                   | 1.771e-23                                      | 7.481e-22                                      |
| Non-alcoholic fatty liver disease (NAFLD) | Cox4i1, Cox4i2, Cox5a, Cox6b1, Cox7a1, Cox7a2, Cox7c, Cox8b, Cyc1, Cycs, Mmp9, Ndufa1, Ndufa11, Ndufa5, Ndufa9, Ndufb11, Ndufb5, Ndufb7, Ndufb8, Ndufs4, Ndufs5, Ndufs6, Ndufs8, Ndufv1, Prkab1, Sdhb, Sdhd, Uqcr10, Uqcr11, Uqcr12, Uqcr13, Uqcr14, Uqcr15, Uqcr16 | 31                          | 149                      | 20.81                                               | 10.97                                                   | 1.960e-19                                      | 6.623e-18                                      |
