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# ABCC1 confers tissue-specific sensitivity to cortisol versus corticosterone; a rationale for safer glucocorticoid replacement therapy

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1    **Title: ABCC1 confers tissue-specific sensitivity to cortisol versus**

2    **corticosterone: a rationale for safer glucocorticoid replacement therapy**

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23

24    **One Sentence Summary:** Corticosterone is excluded from adipose tissue by the trans-

25    membrane transporter ABCC1 and is as effective as cortisol for ACTH suppression, but lacks

26    metabolic adverse effects.

27   **Abstract**

28   The aim of treatment in congenital adrenal hyperplasia is to suppress excess adrenal androgens

29   while achieving physiological glucocorticoid replacement. However, current glucocorticoid

30   replacement regimes are inadequate, because doses sufficient to suppress excess androgens

31   almost invariably induce adverse metabolic effects. Although both cortisol and corticosterone are

32   glucocorticoids that circulate in human plasma, any physiological role for corticosterone has

33   been neglected. In the brain, the ATP-binding cassette transporter ABCB1 exports cortisol but

34   not corticosterone. Conversely, ABCC1 exports corticosterone but not cortisol. We show that

35   *ABCC1* but not *ABCB1* is expressed in human adipose, and that ABCC1 inhibition increases

36   intracellular corticosterone but not cortisol and induces glucocorticoid-responsive gene

37   transcription, in human adipocytes. Both C57Bl/6 mice treated with the ABCC1 inhibitor

38   probenecid and FVB mice with deletion of *Abcc1* accumulated more corticosterone than cortisol

39   in adipose after adrenalectomy and corticosteroid infusion. This accumulation was sufficient to

40   increase glucocorticoid-responsive adipose transcript expression. In human adipose tissue, tissue

41   corticosterone concentrations were consistently low, and *ABCC1* mRNA was upregulated in

42   obesity. To test the hypothesis that corticosterone effectively suppresses ACTH without the

43   metabolic adverse effects of cortisol, we infused cortisol or corticosterone in patients with

44   Addison's disease. ACTH suppression was similar, but subcutaneous adipose transcripts of

45   glucocorticoid-responsive genes was higher after cortisol than corticosterone. These data indicate

46   that corticosterone may be a metabolically favorable alternative to cortisol for glucocorticoid

47   replacement therapy when ACTH suppression is desirable, as in congenital adrenal hyperplasia,

48   and justify development of a pharmaceutical preparation.

**Introduction**

Congenital adrenal hyperplasia (CAH) is characterized by impaired adrenal steroidogenesis, with glucocorticoid deficiency resulting in reduced suppression of adrenocorticotrophic hormone (ACTH), and hence ACTH-dependent adrenal androgen excess. Current treatment guidelines recommend cortisol (hydrocortisone) as the treatment of choice, but recent studies have found that the doses required to suppress adrenal androgen production are usually associated with adverse effects (*1, 2*), notably in adipose tissue where chronic glucocorticoid excess promotes obesity and associated metabolic dysfunction (*3*). The ideal treatment would have higher potency in suppressing ACTH and lower potency as a glucocorticoid acting in peripheral tissues. Here, we provide a rationale and proof of concept for using corticosterone therapy as an alternative to hydrocortisone (cortisol).

A neglected characteristic of the hypothalamus-pituitary-adrenal (HPA) axis is that many species produce two glucocorticoids, cortisol and corticosterone, unlike rats and mice that lack 17-hydroxylase (CYP17) in their adrenals and therefore secrete only corticosterone. Although results of human glucocorticoid receptor binding studies are inconsistent (*4, 5*), both glucocorticoids appear to have similar affinities for mineralocorticoid receptors (*6*) and are subject to similar metabolism (*7*). However, there is emerging evidence for tissue-specific responses to cortisol and corticosterone, mediated by differential susceptibility to transmembrane transport by ATP-binding cassette (ABC) transporters (*8-12*). In rodents, ABCB1 (also known as multidrug resistance protein 1, MDR1, or p-glycoprotein) exports cortisol but not corticosterone across the blood-brain barrier (*10, 12*). Disproportionately high corticosterone concentrations in cerebrospinal fluid and brain tissue suggest that the same

71 mechanism operates in humans (*10, 13*). Another transporter, ABCC1 (or multidrug

72 resistance-related protein 1, MRP1), exports corticosterone but not cortisol in vitro *(11)*. We

73 show expression of ABCC1 but not ABCB1 in human adipose tissue, report in vitro and in

74 vivo data supporting the hypothesis that adipose tissue excludes corticosterone and is

75 preferentially sensitive to cortisol, and provide proof of concept that corticosterone may be a

76 useful therapy especially when suppression of ACTH is desirable, as in CAH.

77    **Results**

78    *ABCC1 but not ABCB1 is expressed in human adipose*

79    In a transcriptomic analysis of ABC transporters in healthy men, *ABCC1* but not *ABCB1* was

80    highly expressed in subcutaneous adipose tissue (Figure 1A and figure S1). This pattern was

81    confirmed by conventional reverse transcriptase polymerase chain reaction amplification in

82    human adipose tissue and in differentiated SGBS human adipocytes (Figure 1B) (*14*), in which

83    we also confirmed that ABCC1 protein and mRNA are regulated in parallel, for example when

84    we induced ABCC1 by incubation with lipopolysaccharide (figure S2). However, in murine

85    adipose tissue, both ABCB1 and ABCC1 were expressed (Figure 1C). Publicly available datasets

86    do not provide sufficient data to test for patterns of ABCB1 and ABCC1 expression in multiple

87    species, but we also found selective adipose expression of ABCC1 but not ABCB1 in horse,

88    another species in which both cortisol and corticosterone are in the circulation (Figure 1D).

89    *ABCC1 preferentially exports corticosterone and confers sensitivity to cortisol in human*

90    *adipocytes in vitro*

91    After incubation of SGBS adipocytes with cortisol or corticosterone for 24 hours, removal of

92    extracellular steroid resulted in lower intracellular concentrations of corticosterone compared to

93    those of cortisol over the subsequent 24 hours (Figure 2A), suggesting active preferential export

94    of corticosterone. This was confirmed and attributed to ABCC1 using the inhibitors MK571 (*15*)

95    and probenecid (*11*). MK571 concentrations (10 μM) sufficient to inhibit ABCC1-dependent

96    export of calcein by ~40% in SGBS cells (figure S3A) (*16*) increased accumulation of

97    intracellular deuterated corticosterone assessed qualitatively using coherent anti-Stokes Raman

98    scattering (CARS) microscopy (Figure 2B). Similarly, both MK571 and probenecid increased

99    intracellular tritiated corticosterone much more so than tritiated cortisol (Figure 2C). Effects of

100   MK571 and probenecid on corticosterone retention were time-dependent (figure S3B) and dose-

101   dependent (figure S3C,D).

102   To test the consequences of ABCC1-mediated steroid transport for glucocorticoid receptor (GR)

103   activation, we used A549 human epithelial cells (which express ABCC1 and GR) transfected

104   with a glucocorticoid-responsive MMTV-luciferase reporter (*17*). The response was greater to

105   cortisol than corticosterone without inhibition of ABCC1, and MK571 potentiated luciferase

106   induction by corticosterone but not cortisol (figure S4A). In differentiated SGBS adipocytes, we

107   tested a panel of known GR-responsive gene transcripts to determine a suitable candidate for

108   assessing acute cortisol versus corticosterone sensitivity (Figure 2D). Of these, only *PER1* was

109   induced acutely (2 hours) by glucocorticoids, consistent with its rapid response (*18*) and high

110   sensitivity (*19*) in other systems. We showed greater sensitivity to cortisol than corticosterone for

111   induction of *PER1* transcription (Figure 2D). MK571 directly increased the amount of this

112   transcript (figure S4B), so probenecid was used alone as an ABCC1 inhibitor, reversing the

113   differential sensitivity to cortisol over corticosterone (Figure 2D). Similar results were obtained

114   after 24 hour incubations (figure S4C). Finally, to confirm whether adipocytes are functionally

115   more sensitive to cortisol than corticosterone, SGBS preadipocytes were differentiated in the

116   presence of glucocorticoid; cortisol but not corticosterone induced marked triglyceride

117   accumulation over 21 days (Figure 2E).

118

119  *Genetic disruption or pharmacological inhibition of Abcc1 in mice results in preferential*

120  *accumulation of corticosterone in adipose tissue*

121  Male control mice ($Abcc1^{+/+}$) and mice deficient in Abcc1 ($Abcc1^{-/-}$) on a FVB genetic

122  background were adrenalectomized and infused with corticosterone and cortisol by subcutaneous

123  osmotic minipump for 7 days, followed by measurement of steroids in plasma and tissue by

124  liquid chromatography tandem mass spectrometry (LC-MS/MS). Plasma analysis (Figure 3A)

125  indicated impaired clearance of glucocorticoids in $Abcc1^{-/-}$ mice, with elevated steady state

126  concentrations of corticosterone and a trend to elevated cortisol compared with control mice, but

127  there was no difference in the circulating corticosterone:cortisol ratio. In the brain (Figure 3B),

128  corticosterone concentrations were higher in $Abcc1^{-/-}$ mice than controls, consistent with

129  increased plasma concentrations, and cortisol was low in both groups, consistent with exclusion

130  of cortisol by ABCB1 (*10*). However, there was no effect of ABCC1 deficiency on brain

131  corticosterone:cortisol ratio. In contrast, in subcutaneous adipose tissue (Figure 3C), *Abcc1*

132  deficiency resulted in an elevated corticosterone:cortisol ratio, with a marked increase in

133  corticosterone but not cortisol concentrations.

134  To assess if pharmacological inhibition of ABCC1 mimicked the effects of genetic deletion on

135  adipose glucocorticoid concentrations, we treated mice with probenecid. Male C57Bl/6 mice

136  underwent the same adrenalectomy and steroid infusion protocol as $Abcc1^{+/+}$ and $Abcc1^{-/-}$ mice,

137  and were treated with probenecid (150 mg/kg/day) or vehicle (saline). Probenecid did not alter

138  corticosterone or cortisol concentrations in the plasma (Figure 3D) or in the brain (Figure 3E);

139  however, consistent with the results from $Abcc1^{-/-}$ mice, probenecid increased corticosterone but

140  not cortisol concentrations in subcutaneous adipose tissue (Figure 3F).

141    To test the metabolic consequences of the increase in intra-adipose corticosterone concentrations

142    with ABCC1 inhibition, we repeated the experiment with probenecid, adding an additional

143    control group with no glucocorticoid infusion, and assessed adipose tissue for glucocorticoid-

144    responsive gene transcripts (Figure 4). Plasma corticosterone and cortisol concentrations were

145    unaffected by probenecid in corticosteroid-infused groups, as before (Figure 4A), and below the

146    lower limit of detection (0.5 ng/ml) in vehicle-treated adrenalectomized animals. Corticosteroid

147    infusion reduced weight gain during the 7-day treatment (Figure 4B) and induced adipose

148    expression of *Per1, Atgl, Hsl,* and *Lpl* (Figure 4C); the effects on body weight and adipose *Per1*,

149    *Atgl,* and *Lpl* were potentiated by probenecid (Figure 4B,C).

150    *Corticosterone concentrations are low in human adipose, and not increased in obesity*

151    To compare endogenous cortisol and corticosterone concentrations in adipose tissue, and to test

152    whether downregulation of ABCC1 might enhance corticosterone action in adipose tissue in

153    obesity, we collected subcutaneous and visceral adipose biopsies during elective abdominal

154    surgical procedures from 6 lean and 6 obese patients, with characteristics shown in table S1.

155    *ABCC1* mRNA transcripts were increased in obese subjects in both subcutaneous and visceral

156    adipose (Figure 5A). Adipose tissue cortisol was readily measured in both adipose depots, as

157    previously described (*20*), and not different between lean and obese subjects (Figure 5B).

158    Corticosterone concentrations in lean and obese subjects were below or near the limit of

159    detection by LC/MS-MS in both visceral and subcutaneous adipose tissue (Figure 5B).

160    *Human adipose is less sensitive to corticosterone than cortisol*

161    Having established that selective expression of ABCC1 protects human adipose tissue from

162    corticosterone, we then tested whether corticosterone is less potent than cortisol at inducing

163    metabolic effects in adipose. This was achieved using doses of cortisol and corticosterone

164 equipotent for ACTH suppression. We performed a randomized single-blind crossover study in

165 patients with Addison's disease using sequentially increasing steady state infusions of deuterated

166 glucocorticoids, D8-corticosterone and D4-cortisol, which can be distinguished from residual

167 endogenous glucocorticoids by mass spectrometry (*21*). Characteristics of study participants are

168 summarized in table S2. Circulating concentrations of cortisol (sum of cortisol, D4-cortisol, and

169 its active metabolite D3-cortisol) and corticosterone (D8-corticosterone alone; endogenous

170 corticosterone was undetectable) indicated that serially increasing steady state concentrations

171 were achieved as intended (Figure 6A). Total steady state concentrations tended to be higher for

172 cortisol than corticosterone, although the differences were not statistically significant and there

173 were no differences in free cortisol and corticosterone measured after equilibrium dialysis (in

174 samples pooled between 260 and 330 min of infusion, free cortisol = 38.7 ± 8.2 nM and free

175 corticosterone = 37.2 ± 4.7 nM). Neither ACTH concentrations at baseline nor their suppression

176 during glucocorticoid infusion were different between cortisol and corticosterone (Figure 6B,C;

177 Table 1). Amongst biochemical markers of glucocorticoid action – glucose, insulin, glycerol, and

178 free fatty acids – only free fatty acids rose during steroid infusion (Table 1), perhaps because the

179 infusions were short and the glucocorticoid concentrations achieved were within the

180 physiological range (*22*). Insulin concentrations fell, paradoxically, during steroid infusions.

181 None of these plasma biochemical markers differed between D8-corticosterone and D4-cortisol

182 infusion (Table 1). However, in subcutaneous adipose biopsies obtained at the end of infusions,

183 the acutely responsive glucocorticoid-responsive transcript *PER1* was substantially higher and

184 *LPL* also modestly higher after D4-cortisol than D8-corticosterone infusion (Figure 6D).

185    **Discussion**

186    Our findings shed light on tissue-specific responsiveness to cortisol and the often-neglected

187    'second' glucocorticoid corticosterone, mediated by local expression of steroid-selective ABC

188    transporters. In combination with tissue-specific expression of either mineralocorticoid or

189    glucocorticoid receptors and tissue-specific intracellular metabolism of corticosteroids, the

190    availability of alternate endogenous glucocorticoids that are differentially transported out of

191    target cells provides a mechanism for subtle control of the otherwise highly conserved pathway

192    of glucocorticoid action. Moreover, as summarized in the schematic in Figure 7, our data show

193    how this insight can be exploited to develop corticosterone as a potentially safer alternative to

194    cortisol for glucocorticoid replacement therapy when ACTH suppression is desirable.

195        For in vitro studies, we used SGBS adipocytes, an established model of human

196    adipocytes (*14*), to show that in the absence of ABCB1, endogenous ABCC1 expression is

197    sufficient to reduce intracellular corticosterone but not cortisol concentrations. An equilibrium in

198    intracellular cortisol and corticosterone concentrations was reached after approximately 4 hours

199    in adipocytes. Our CARS microscopy data suggest that glucocorticoids co-localize with lipid

200    droplets within adipocytes, potentially resulting in their intracellular retention and explaining the

201    slow turnover of cortisol in adipose tissue in vivo *(20)*. The magnitude of the effect of ABCC1

202    on steroid export appears to be substantial, with a 3-4 fold increase in intracellular corticosterone

203    when ABCC1 is inhibited in SGBS cells, and a 2-fold increase in adipose corticosterone in vivo

204    in mice. In a physiological context, if the plasma glucocorticoid pool is comprised of 90-95%

205    cortisol and 5-10% corticosterone, as reported in previous studies (*10, 13, 23-32*) (table S3), the

206    total (cortisol plus corticosterone) intra-adipose glucocorticoid pool may be reduced by as much

207    as ~7% as a result of ABCC1 activity. In a pathological context, we hypothesized that reduction

208    of adipose ABCC1 in obesity might contribute to metabolic dysregulation through increased

209    intra-adipose corticosterone. However, *ABCC1* expression was increased in obesity, suggesting a

210    potential protective mechanism to limit adipose exposure to glucocorticoids, which is considered

211    to be beneficial in obesity (*3*). Indeed, adipose glucocorticoid concentrations were consistent

212    with ABCC1 acting as constitutive barrier to maintain very low corticosterone concentrations in

213    human adipose tissue, even in obese individuals. In a pharmacological context, such as if

214    corticosterone were used as glucocorticoid replacement therapy, the consequences of ABCC1

215    activity for the intra-adipose glucocorticoid pool could be much greater. We tested this in vivo in

216    mice and humans.

217         Our findings in vivo support our cell-based work, with both genetic deletion and

218    pharmacological inhibition of ABCC1 resulting in preferential accumulation of corticosterone

219    over cortisol in the adipose tissue. Because 17α-hydroxylase, which is necessary to produce

220    cortisol, is not expressed in mouse adrenals, we studied this in adrenalectomized mice infused

221    with equal concentrations of both glucocorticoids. Plasma steroid concentrations achieved during

222    infusion were similar within strains, but slightly higher in C57Bl6 than FVB mice, potentially

223    reflecting strain-specific differences in glucocorticoid clearance. *Abcc1*$^{-/-}$ mice, but not mice

224    treated with probenecid, showed higher steady-state plasma concentrations of cortisol and

225    corticosterone compared to wild type controls, implicating ABCC1 in total glucocorticoid

226    clearance; this effect, however, appears to require total loss of *ABCC1,* as it was not observed

227    with a competitive inhibitor, probenecid. Of note, the only spontaneous phenotype reported in

228    *Abcc1<sup>-/-</sup>* mice is a reduced susceptibility to inflammation (*33*), which may be mediated by

229    systemic or intracellular corticosterone excess, but this has not been studied. Using probenecid,

230    we showed that the increase in intra-adipose corticosterone concentrations with ABCC1

231    inhibition is sufficient to potentiate the induction of key lipolytic genes (*Atgl* and *Hsl)* and

232    prevent weight gain.

233        To investigate the physiological and therapeutic implications of these findings, we

234    performed a study in patients with Addison's disease, who lack endogenous glucocorticoids. We

235    selected patients with Addison's disease rather than with CAH to avoid confounding effects of

236    high androgen concentrations in the adipose tissue. Corticosterone is only available as an

237    experimental tool for infusion and not as a licensed pharmaceutical, therefore only short-term

238    manipulation was possible. At similar concentrations of free cortisol and corticosterone, we

239    found equipotent suppression of ACTH, but substantially greater gene transcript induction in

240    adipose tissue by cortisol. In adipose, cortisol induced greater transcript expression than

241    corticosterone not only for *PER1*, which is known to be rapidly induced by glucocorticoids (*18*)

242    and sensitive at low concentrations which do not alter other glucocorticoid-responsive genes

243    (*19*), but also for *LPL,* a key enzyme involved in adipose triglyceride uptake. The lack of

244    differences in circulating metabolic markers is likely attributable to the short duration of

245    infusions and the glucocorticoid concentrations within the physiological range (*22*); previous

246    studies have that demonstrated plasma glucose, insulin, and glycerol show responses only to

247    several hours of 'physiological' glucocorticoid infusion (*34, 35*). It remains possible that there

248    are intrinsic differences in transcriptional response to cortisol and corticosterone in human

249    adipose tissue, even when the same concentrations of steroid are present, but this seems unlikely

250    given our findings in SGBS adipocytes that cortisol is no more potent than corticosterone when

251 ABCC1 is inhibited. Despite our confirmation that cortisol concentrations are disproportionately

252 low in the mouse brain, and earlier observations of relatively low cortisol in human brain and

253 cerebral spinal fluid (*10, 13*), we did not find that corticosterone was more effective than cortisol

254 in suppressing ACTH. This may reflect the major feedback signal being mediated in the pituitary

255 rather than higher centers within the brain, under non-stressed conditions. Nonetheless, the

256 discrepancy between suppression of ACTH and induction of adipose *PER1* and *LPL* by cortisol

257 versus corticosterone supports our interpretation that adipose tissue is physiologically more

258 responsive to cortisol than corticosterone, and that this can be exploited therapeutically.

259 Synthetic glucocorticoids in common use as glucocorticoid therapy, specifically

260 prednisolone and dexamethasone, are not transported by ABCC1 (*11*) and so are likely to access

261 adipose tissue similarly to cortisol. To validate and exploit these findings, it will therefore be

262 important to develop a suitable pharmaceutical preparation of corticosterone. With longer term

263 therapy, corticosterone and cortisol effects can be compared in conditions requiring effective

264 ACTH suppression such as CAH, Nelson's syndrome, and glucocorticoid-suppressible

265 hyperaldosteronism. Although we have focused here on adipose tissue, differential expression of

266 ABCB1 and ABCC1 may determine tissue-specific responses to corticosterone and cortisol in

267 other tissues as well. Adverse effects will need to be compared not only for metabolism and

268 obesity, but also for immune suppression and osteoporosis. Nevertheless, our findings suggest

269 that the substantial investment required to develop a corticosterone-based drug product is

270 worthwhile.

271

272    **Materials and Methods**

273    *Study design*

274    To investigate the role of adipose ABCC1 regulating cortisol versus corticosterone action, we

275    conducted studies in cells, mice, and humans, as detailed below. In vitro experiments were

276    performed in triplicate with the number of experiments and outcomes defined in figure legends.

277    For in vivo studies in mice, experiments were approved by the institutional ethical committee

278    and conducted under UK Home Office license in male mice aged 10-12 weeks at the start of the

279    experiment. Sample sizes were chosen for 80% power to detect magnitudes of difference inferred

280    from in vitro experiments at $P<0.05$, with the number of mice and outcomes defined in figure

281    legends. Mice of each genotype were randomly assigned to interventions within each

282    experiment. The studies in humans were conducted with approval from the South East Scotland

283    Research Ethics Committee and NHS Lothian Research and Development (13/SS/0210 for

284    Addison's disease study and 10/S1102/39 for surgical adipose tissue collection study), and with

285    written informed consent of participants. The surgical adipose tissue collection study was a case-

286    control design comparing adipose tissue mRNA amounts in lean and obese participants randomly

287    selected from those undergoing surgery. An initial exploratory study was conducted in n=6 per

288    group and showed results contradicting the hypothesis that *ABCC1* transcripts are decreased in

289    obesity, so the study was not expanded. For the Addison's disease study, eligible patients

290    participated in a single-blind randomized crossover study comparing infusions of deuterated

291    corticosterone and deuterated cortisol for effects on ACTH, adipose tissue mRNA, and

292    circulating metabolic indices. Blinding of the clinical investigator was impractical because of the

293 requirements for administration of different loading and infusion doses of cortisol and

294 corticosterone, but samples were processed in the laboratory with blinded codes and only

295 decoded for statistical analysis; patients were blinded to treatment. Sample size was calculated

296 for 90% power to detect 20% difference in ACTH at $p<0.05$. Inclusion/exclusion criteria for each

297 study are detailed below. There were no dropouts, and no outliers were excluded from any

298 studies.

299 *Human adipose tissue microarray*

300 Affymetrix microarray data were obtained from subcutaneous adipose biopsies of 9 healthy men,

301 aged $36.6 \pm 2.1$ years with body mass indices $29.2 \pm 2.1$ kg/m$^2$ (*36*). Probes for transcripts

302 encoding ABC transporters were selected and ranked by transcript intensity (log$^2$ transformed).

303 *Polymerase chain reaction*

304 Human tissue biobank cDNA (Primerdesign Ltd) was used as a template for the identification of

305 ABC transporters in human tissue of interest. Equine peri-renal adipose tissue collection from

306 clinical cases euthanized for reasons other than endocrine disease/systemic inflammation was

307 approved by the University of Edinburgh Veterinary ethics and research committee. Where

308 applicable, total RNA extraction was carried out by centrifugation in Qiazol lysis reagent using

309 an RNeasy mini kit (Qiagen), according to the manufacturer's instructions. RNA integrity was

310 checked on a 1% agarose gel, after which cDNA (500 ng RNA/reaction) was synthesized using a

311 high capacity cDNA kit (Invitrogen), according to the manufacturer's instructions. cDNA was

312 used as a template for specific primers (Invitrogen; table S4) for RT-PCR and amplified using

313 GoTaq DNA polymerase (Promega) as per the manufacturer's instructions, on a Techne

314 thermocycler (95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; 72 °C

315 for 5 min). Products were subjected to gel electrophoresis on a 2% agarose gel in TAE buffer

316 (50x stock buffer: 2 M Tris Base, 1 M glacial acetic acid, 100 mM disodium EDTA) containing

317 GelRed (Cambridge Bioscience). Gels were imaged using a Gel-Doc system (Uvitec). Product

318 size was confirmed against a 100 bp DNA ladder (Invitrogen).

319 **In vitro studies**

320 *SGBS cell culture*

321 Human preadipocyte Simpson-Golabi-Behmel syndrome (SGBS) cells were a kind gift from

322 Martin Wabitsch, University of Ulm, Germany (*14*). Cells were maintained in high-glucose

323 DMEM-F12 (Lonza), supplemented with FBS (10% v/v), penicillin (100 IU/ml), streptomycin

324 (100 IU/ml), biotin (33 μM), and pantothenic acid (17 μM) at 37°C in 5% $CO_2$. Differentiation

325 was induced as previously described (*37*). Cells were cultured in steroid-free medium for 24

326 hours before experimentation.

327 *Primary cell culture*

328 Subcutaneous adipose tissue samples were obtained from patients undergoing elective abdominal

329 surgery at the Royal Infirmary of Edinburgh. Upon tissue collection, adipose was digested, and

330 the stromal vascular fraction was isolated and differentiated as previously described (*38*). In

331 brief, after removal of connective tissue and blood vessels, adipose tissue was digested in

332 collagenase type I (615 U/g tissue, 90 min, 37 °C). After overnight plating in high-glucose

333 DMEM-F12 supplemented with FBS (10% v/v), penicillin (100 IU/ml), streptomycin (100

334 IU/ml), biotin (33 μM), and pantothenic acid (17 μM), cells were differentiated for 3 days using

335 the above medium without serum, but with addition of triiodothyronine (1 nM), transferrin (10

336 μg/ml), insulin (66 nM), IBMX (500 μM), dexamethasone (1 μM), and rosiglitazone (10 μM).

337 From day 4 onwards, cells were maintained in this differentiation medium but without IBMX,

338 dexamethasone, or rosiglitazone.

*Tritiated glucocorticoid retention assays*

340  To measure uptake and retention of steroids, SGBS adipocytes were incubated in the presence of

341  either corticosterone or cortisol (20 nM 1,2,6,7-$^3$H$_4$-steroid, 480 nM unlabeled steroid) for 1, 4,

342  8, and 24 hours before being washed in PBS, lysed in cellular lysis buffer (0.5% SDS), mixed

343  with Prosafe FC+ liquid scintillation cocktail (Meridan Biotechnologies), and read for 1 min on a

344  β-scintillation counter. A separate batch of SGBS adipocytes were incubated with steroids for 24

345  hours as described above, then washed in PBS before fresh steroid-free medium was added to

346  each well and the decline in intracellular $^3$H-steroid content measured at 1, 4, 8 and 24 hours.

347  To assess the effect of ABCC1 inhibition, SGBS adipocytes were pre-incubated for 1 hour in the

348  presence of the indicated concentrations of MK-571 (Cayman Chemical), probenecid

349  (Invitrogen), or vehicle (DMSO), before being incubated for a further 24 hours in the presence of

350  inhibitor or vehicle and either corticosterone or cortisol (20 nM $^3$H-steroid, 480 nM unlabeled

351  steroid). Cells were then washed in PBS, lysed in cellular lysis buffer, mixed with liquid

352  scintillation cocktail, and read for 5 min on a β-scintillation counter.

353

354  *Quantification of glucocorticoid-sensitive mRNA transcripts*

355  In accordance with the time course of the clinical study, we performed an acute (2 hour)

356  treatment in SGBS adipocytes and an extended (24 hour) treatment with either vehicle or cortisol

357  (500 nM) to identify acutely upregulated glucocorticoid-responsive transcripts. To determine the

358  effects of ABCC1 inhibition on glucocorticoid-induced transcripts, SGBS adipocytes were pre-

359  incubated for 1 hour in the presence of probenecid (50 μM) or vehicle (DMSO), before a further

360  2 or 24 hours incubation in the presence of probenecid or vehicle and either corticosterone or

361  cortisol (500 nM). Total RNA was extracted from adipocytes in Qiazol lysis reagent using a

362   RNeasy Mini Kit according to the manufacturer's instructions. RNA (250 ng) was reverse

363   transcribed with random primers using the Applied Biosystems High-Capacity cDNA Reverse

364   Transcription kit. Real-time PCR was performed using the Roche LightCycler 480 (Roche

365   Applied Science). Primers (Invitrogen) were designed for use with intron-spanning probes within

366   the Roche Universal Probe Library (UPL). Primer sequences and UPL probe numbers are shown

367   in table S5. Results were corrected for abundance of 18S, which was not affected by treatment.

368   All samples were analyszd in triplicate and amplification curves plotted (y axis fluorescence, x

369   axis cycle number). Triplicates were deemed acceptable if the standard deviation of the crossing

370   point was < 0.5 cycles. A standard curve (y axis crossing point, x axis log concentration) for each

371   gene was generated by serial dilution of cDNAs pooled from different samples, fitted with a

372   straight line, and deemed acceptable if reaction efficiency was between 1.7 and 2.1.

373   *CARS microscopy*

374   Coherent Anti-Stokes Raman Scattering (CARS) microscopy is a non-invasive, label-free

375   imaging technique based on Raman spectroscopy. The experimental setup has been described

376   previously (*39*). Briefly, a pump and tuneable Stokes laser (PicoTrain, High-Q laser and Levante

377   Emerald Optical Parametric Oscillator) provided a specific vibrational coherence resulting in

378   detectable photons which, when combined with a confocal laser scanning inverted microscope

379   (C1 Eclipse, Nikon BV), provided a spatial image. For CARS imaging, the pump and Stokes

380   laser beams were tuned such that the frequency difference would correspond to a specific Raman

381   vibration.

382   SGBS  adipocytes  were  incubated  for  24  hours  in  glass-bottomed  dishes  with

383   $2,2,4,6,6,17\alpha,21,21-^2H_8$-corticosterone (D8-corticosterone, 10 µM; Cambridge Isotopes) in the

384   presence or absence of MK-571 (10 µM). For CARS imaging, the pump laser was tuned to 816.8

385     nm (12243 cm$^{-1}$) and Stokes laser to 1064 nm (9398.5 cm$^{-1}$) to obtain a CARS signal at 663 nm

386     (2845 cm$^{-1}$) corresponding to the vibration of CH$_2$ in lipids (*39*). After initial documentation of

387     Raman spectra to identify optimal wavelength (figure S5), excitation of the Raman vibration of

388     the C-D bond in D8-corticosterone was achieved by adjusting the pump laser to 868.4 nm

389     (11524.5 cm$^{-1}$), resulting in a CARS signal at 733 nm (2126 cm$^{-1}$), which was in a region of the

390     Raman spectrum with low background signal. Images were processed using Nikon EZ-C1 3.4

391     software.

392     *Protein analysis*

393     Total protein extracts from cells were prepared in RIPA buffer (Santa Cruz). Samples were

394     sonicated and centrifuged (13,000 x g, 15 min, 4 °C) before performing the BCA assay, adding

395     6X Laemmli sample buffer (LSB), boiling, and SDS-PAGE resolution. ABCC1 (Enzo Life

396     Sciences, ALX-801-007) and HSP90 (Santa Cruz, sc-7947) were detected on Western blots

397     using commercial antibodies.

398

399     *Oil Red O staining and lipid quantification*

400     To assess potential differing effects of cortisol and corticosterone on adipocyte differentiation,

401     SGBS adipocytes were stimulated to undergo differentiation with or without the substitution of

402     cortisol in the differentiation cocktail (*37*) for corticosterone. Accumulation of lipid during

403     adipogenesis was visualized by Oil Red O (Sigma-Aldrich). Cells were washed twice with PBS

404     and fixed with formalin (10% v/v) for 60 min, followed by a wash with isopropanol (60% v/v).

405     Working Oil Red O solution was added to cells for 10 min, followed by 4 washes with H$_2$O.

406     Cells were air-dried and dye extracted with isopropanol (100%). Absorbance of extracts were

407     measured at 500 nm wavelength in an OPTImax microplate reader (Molecular Devices).

408 *Calcein-AM Assay*

409 Calcein–acetoxymethyl ester (AM) is a non-fluorescent, hydrophilic, cell membrane permeable

410 molecule which is converted to fluorescent, hydrophilic calcein by intracellular esterases (*16*).

411 Both calcein-AM and calcein are substrates for ABCC1, thus intracellular fluorescence is

412 inversely proportional to ABCC1 activity. SGBS adipocytes were washed with PBS and pre-

413 incubated for 1 hour in the presence of MK-571 (10 µM) or vehicle (DMSO), before being

414 incubated for a further 5 hours in the presence of inhibitor or vehicle and calcein-AM (1 µM;

415 Invitrogen). Cells were washed three times in PBS, then excitation absorbance was measured at

416 494 nm and emission absorbance was measured at 517 nm in an Infinite M1000 plate reader

417 (Tecan). Data are expressed as percentage fluorescence of untreated control.

418 *Luciferase reporter assay*

419 Human epithelial A549 cells were grown in DMEM (Lonza), supplemented with FBS (10% v/v),

420 penicillin (100 IU/ml), and streptomycin (100 IU/ml). Cells were seeded at $2 \times 10^5$/ 35 mm well.

421 After overnight incubation, medium was replaced with Opti-MEM (Lonza), and cells were

422 transfected using Lipofectamine 2000 (Invitrogen) with a total of 2 µg DNA comprising 1 µg

423 pMMTV-LTR-Luc (*40*) and 1 µg pKC275 (encoding β-galactosidase as internal control). After

424 overnight incubation, medium was replaced with steroid-free medium, and cells were treated for

425 1 hour with MK-571 (10 µM) before a 24-hour incubation with inhibitor and either

426 corticosterone or cortisol (500 nM). Luciferase and β-galactosidase activities were measured in

427 cell lysates as described previously (*41*). β-Galactosidase activity was assayed using a Tropix

428 Galacto Light Plus kit (Applied Biosystems). All transfections were carried out in triplicate, and

429 the mean ratio of luciferase/ β-galactosidase activities was calculated.

430 **Animal studies**

431 *Animals*

432 Male *Abcc1* knockout mice (*Abcc1$^{-/-}$*) and FVB controls (*Abcc1$^{+/+}$*) were purchased from

433 Taconic. Male C57Bl/J mice were purchased from Charles River. Mice were bilaterally

434 adrenalectomized under fluorothane anesthesia. After surgery, drinking water was replaced with

435 0.9% NaCl, and animals were allowed to recover for 7 days. An osmotic mini-pump (Alzet

436 Model 2001; 1 µl/hr) delivering 250 µg/day of corticosterone and 250 µg/day cortisol or vehicle

437 (DMSO: propylene glycol; 50:50 v/v) was inserted subcutaneously under anesthesia and left in

438 place for 7 days. *Abcc1$^{+/+}$* and *Abcc1$^{-/-}$* mice received no further treatment. C57Bl/6 mice were

439 given daily s.c. injections of probenecid (150 mg/kg) or vehicle (saline). This dose was

440 previously reported to inhibit transporter activity in vivo (*42*). 7 days after osmotic pump

441 implantation, animals were culled by decapitation. Plasma was extracted from trunk blood and

442 stored at -20 °C. Tissue was extracted and stored at -80 °C.

443

444 *Plasma and tissue steroid extraction and LC-MS/MS quantification*

445 Steroids were extracted from plasma by liquid-liquid extraction (chloroform, 10:1). Briefly,

446 plasma (100 µl) was enriched with internal standard (D4-cortisol and epi-corticosterone, 25 ng

447 each). 1 ml chloroform was added and vortexed. Supernatant was reduced to dryness under

448 oxygen-free nitrogen (OFN) at 60°C and reconstituted in mobile phase [70 µl water/acetonitrile

449 (70:30, v/v)]. Steroids were extracted from brain and adipose as previously described (*20*), with

450 the substitution of D4-cortisol and D8-corticosterone as internal standards. Steroids were

451 extracted from brain by homogenizing whole brain in methanol:acetic acid (100:1 v/v; 10 ml)

452 and centrifuging (5000x g, 10 min, 4°C). The supernatant was reduced to dryness under OFN at

453    60°C, reconstituted in methanol:dichloromethane:water (7:2:1, v/v; 3 ml), and enriched with

454    internal standard (D4-cortisol and D8-corticosterone, 25 ng each). Samples were passed through

455    a diethylaminohydroxypropyl Sephadex LH-20 anion exchange column (GE Healthcare).

456    Columns were washed with methanol:dichloromethane:water (7:2:1, v/v; 2 ml) and

457    methanol:dichloromethane:water (2:2:1, v/v; 1 ml). All flow-through/wash was collected and

458    reduced to dryness under OFN at 60°C. Samples were reconstituted in methanol:acetic acid

459    (100:1 v/v; 2 ml), and water (2 ml) was added. Samples were passed through pre-conditioned C-

460    18 Bond-Elut columns (Agilent Technologies). Columns were washed with water (2 ml),

461    methanol:water (50:50 v/v; 3 ml), and hexane:ethyl acetate (5:1 v/v; 2 ml). Samples were eluted

462    in ethyl acetate (2 ml), dried under OFN at 60°C and reconstituted in mobile phase [60 μl

463    water/acetonitrile (70:30, v/v)].

464    Quantitative analysis of steroids was carried out by liquid chromatography tandem mass

465    spectrometry (LC-MS/MS). Chromatographic separation was achieved using a Waters Acquity

466    UPLC system, with detection on an ABSciex QTRAP 5500 mass spectrometer operated with

467    Analyst software version 1.6.1. The mass spectrometer was operated using the Turbospray Ion

468    source, with nitrogen as the source, curtain and collision gas (40 and 60 psi, medium), source

469    temperature of 550ºC, spray voltage of 4.5 kV, and an entrance potential of 10 V. Compound-

470    specific tuning was performed using methanolic solutions of steroids and isotopically labeled

471    internal standards. The protonated molecular ions were subjected to collision-induced

472    dissociation, and the most abundant precursor-product transitions were selected; $m/z$ 347.2 →

473    91.1, 121.1 at 69 V, $m/z$ 355.1 → 337.0 at 19 V, $m/z$ 363.2 → 121.0, 77.0 at 29 and 101 V, and

474    $m/z$ 367.0 → 121.1 at 29 and 101 V collision energy for corticosterone, D8-corticosterone,

475    cortisol, and D4-cortisol, respectively. Analytes were eluted on a Waters Sunfire C18 column

476    (150 x 2.1 mm; 3.5 μm) at 30°C (injection volume; 30 μL) protected by a Kinetex KrudKatcher

477    at a flow rate of 0.5 mL/min, starting at 30:70 and rising linearly to 90:10 [water+0.1% formic

478    acid (FA):acetonitrile+0.1% FA] by 6 minutes and a total run time, including re-equilibration, of

479    9 minutes. The peak areas were integrated using Xcalibur software (Thermo Electron) and

480    quantified against a calibration curve. Steroid concentrations are presented corrected for total

481    tissue weight or plasma volume.

482    *Quantification of glucocorticoid-sensitive mRNA transcripts*

483    RNA analysis performed was as described above. Primer sequences and UPL probe numbers are

484    shown in table S6. Results were corrected for abundance of the mean combination of *18S* and

485    *Tbp*, which was not affected by treatment.

486

487    **Adipose tissue steroid concentrations and *ABCC1* mRNA in lean and obese humans**

488    We recruited 12 subjects who were at the Royal Infirmary of Edinburgh for elective abdominal

489    surgery for non-malignant disease, and who did not have systemic or local active inflammation.

490    Paired adipose tissue samples were obtained intra-operatively from the subcutaneous and

491    visceral depots, stored on dry ice then at -80C, and extracted for steroid quantification as

492    previously described (*20*) and for RNA analysis as described above.

493

494    **Cortisol versus corticosterone infusion in patients with Addison's disease**

495    *Participants*

496    Patients with Addison's disease were identified from the clinic database of the Edinburgh Centre

497    for Endocrinology and invited to attend a screening visit, where written informed consent was

498    obtained and eligibility assessed by a medical questionnaire, physical examination, and routine

499    blood tests. Inclusion criteria were: a diagnosis of autoimmune Addison's disease and age >18 y.

500    Exclusion criteria were: alcohol intake >28 units/week; abnormal screening blood results (full

501    blood count; renal, liver, and thyroid function tests); pituitary disease; pregnant or breastfeeding;

502    anti-inflammatory glucocorticoid therapy by any route in the preceding 3 months; cardiac, renal

503    or liver failure; uncontrolled hypertension (systolic BP >160 mmHg or diastolic >100 mmHg);

504    blood donation in preceding 3 months (to avoid anemia); or research study participation in

505    preceding 6 weeks.

506    *Study protocol*

507    Participants attended for study visits on two occasions, separated by at least one week.

508    Participants withheld hydrocortisone from 14:00h on the day before each study visit, and omitted

509    fludrocortisone (when prescribed) the morning before the study visit and on the morning of the

510    study visit. They attended the clinical research facility at 08:00h after an overnight fast from

511    22:00h. At $t$ = -15 minutes, intravenous cannulae (18G) were inserted in each antecubital fossa.

512    At $t$ = 0, saline infusion (0.9 %, 125 ml/h) was commenced through the cannula in the left arm,

513    and blood samples collected from the cannula in the right arm.  At $t$ = 60 min, infusion of

514    deuterated glucocorticoid (D8-corticosterone or D4-cortisol) was commenced. Order of steroid

515    infusion was allocated randomly, and study participants were blinded to the order of infusate.

516    9,11,12,12-[$^2$H]$_4$-cortisol    (D4-cortisol)    and    2,2,4,6,6,17,21,21-[$^2$H]$_8$-corticosterone    (D8-

517    corticosterone) were obtained from Cambridge Isotope Laboratories, dissolved in pharmaceutical

518    grade ethanol/water (90:10, v/v) and filtered to form sterile stock solutions, stored (-40 °C) for a

519    maximum of 8 weeks. On study days, D8-corticosterone (4.18 mg/ml) or D4-cortisol (2.5

520    mg/ml) stock solution (5 ml) was dissolved in sodium chloride 0.9% *w/v* (495 ml). At $t$ = 60 min,

521    a priming dose (0.65 μmol D8-corticosterone; 0.23 μmol D4-cortisol) was administered over 4

522  minutes, followed by steady state infusion (27.6 nmol/min D8-corticosterone; 3.7 nmol/min D4-

523  cortisol) for 86 minutes. Further priming doses were administered at $t$ = 150 min (1.95 µmol D8-

524  corticosterone; 0.65 µmol D4-cortisol) followed by constant infusion (111.2 nmol/min D8-

525  corticosterone; 17.2 nmol/min D4-cortisol) until $t$ = 240 min, and at 240 min (3.89 µmol D8-

526  corticosterone; 1.55 µmol D4-cortisol) followed by constant infusion (277.8 nmol/min D8-

527  corticosterone; 51.5 nmol/min D4-cortisol) until $t$ = 330 min.

528  At $t$ = 330 min, a needle aspiration biopsy of subcutaneous abdominal adipose tissue was

529  obtained as previously described (*36*) and stored at -80 ºC.

530  Blood samples were obtained at 10-20 min intervals in potassium EDTA tubes (2.7 ml) pre-

531  chilled on wet-ice and serum gel tubes (9 ml; both Monovette, Sarstedt). Potassium EDTA tubes

532  were centrifuged at 4 ºC within 30 minutes of sampling; serum gel samples were left at room

533  temperature for 30-45 minutes before centrifugation. Serum and plasma was stored at -80 ºC.

534  *Laboratory analyses*

535  In plasma, ACTH was quantified by ELISA (IBL International) within 6 weeks of sampling, and

536  non-esterified fatty acids (NEFAs)(Zen-Bio) and glycerol (Sigma-Aldrich) quantified by

537  colorimetric assays. In serum, glucocorticoids were quantified by LC-MS/MS, insulin by ELISA

538  (DRG Diagnostics), and glucose by colorimetric assay (Cayman Chemical).

539  LC-MS/MS analysis was undertaken as described for animal samples above with the exception

540  that 11α-epimers of corticosterone (epi-corticosterone; $m/z$ 347.2 → 91.1, 121.1 at 69 V) and

541  cortisol (epi-cortisol; ($m/z$ 363.2 → 121.0, 77.0 at 29 and 101 V) were used as internal standards

542  instead of D8-corticosterone and D4-cortisol.

543  To account for any differences in protein binding between cortisol and corticosterone, cortisol

544  and corticosterone isotopologs were also measured by LC-MS/MS after equilibrium dialysis of

545   plasma. To achieve the necessary sensitivity, samples were pooled for the final steady state

546   period from each infusion (260-330 min), and 4 x 1 mL aliquots of plasma were dialyzed into 1.5

547   mL phosphate buffered saline across 12-14 kD dialysis membrane (Medicell) for 16 hours at 37

548   $^{\circ}$C, as previously described (*43*), before LC-MS/MS analysis of the pooled dialysate as above.

549   Real-time qPCR was carried out in adipose tissue as with SGBS cells above.

550   **Statistical analysis**

551   For cell and mouse-based studies, comparisons were performed using two-way ANOVA with

552   Bonferroni post-hoc tests or unpaired Student's t tests as outlined in each figure legend. For the

553   Addison's disease study, data from each steady state period (80-140 min; 160-240 min; 260-330

554   min) were averaged, and comparisons between D4-cortisol and D8-corticosterone and the

555   interaction with changes over time were performed by two-way ANOVA. Adipose tissue data

556   were compared with paired Student's t tests. P values for statistically significant differences are

557   presented in table S7.

558    **Supplementary Materials**

559    *Supplementary figures*

560    Fig. S1. Whole gel PCR images of ABC transporter expression in tissues and cells.

561    Fig. S2. Correlation of *ABCC1* mRNA and protein levels in human adipocytes.

562    Fig. S3. ABCC1 inhibition in SGBS adipocytes.

563    Fig. S4. Effects of ABCC1 inhibition on GR-mediated transcription.

564    Fig. S5. Optimisation of CARS microscopy for detection of intracellular D8-corticosterone.

565

566    *Supplementary tables*

567    Table S1. Characteristics of lean and obese study participants providing adipose biopsy samples

568    during surgery.

569    Table S2. Characteristics of Addison's study participants.

570    Table S3. Summary of studies describing plasma corticosterone and cortisol concentrations in

571    healthy subjects.

572    Table S4. Primer sequences for PCR and corresponding expected product size.

573    Table S5. Human primer sequences for qPCR and corresponding probe number from Roche

574    Universal Probe Library (UPL).

575    Table S6. Murine primer sequences for qPCR and corresponding probe number from Roche

576    Universal Probe Library (UPL).

577    Table S7. Summary table of exact *P* values (provided as an Excel file).

578     **References and notes**

579     1.     T. S. Han, R. H. Stimson, D. A. Rees, N. Krone, D. S. Willis, G. S. Conway, W. Arlt, B. R.

580            Walker, R. J. Ross, E. United Kingdom Congenital adrenal Hyperplasia Adult Study,

581            Glucocorticoid treatment regimen and health outcomes in adults with congenital adrenal

582            hyperplasia. *Clin Endocrinol (Oxf)* **78**, 197 (2013).

583     2.     T. S. Han, B. R. Walker, W. Arlt, R. J. Ross, Treatment and health outcomes in adults with

584            congenital adrenal hyperplasia. *Nat Rev Endocrinol* **10**, 115 (2014).

585     3.     B. R. Walker, Cortisol - cause and cure for metabolic syndrome? *Diabetic Medicine* **23**,

586            1281 (2006).

587     4.     G. Giannopoulos, D. Keichline, Species-related differences in steroid-binding specificity of

588            glucocorticoid receptors in lung. *Endocrinology* **108**, 1414 (1981).

589     5.     T. S. Berger, Z. Parandoosh, B. W. Perry, R. B. Stein, Interaction of glucocorticoid

590            analogues with the human glucocorticoid receptor. *The Journal of steroid biochemistry and*

591            *molecular biology* **41**, 733 (1992).

592     6.     J. Arriza, C. Weinberger, G. Cerelli, T. Glaser, B. Handelin, D. Housman, R. Evans,

593            Cloning of human mineralocorticoid receptor complementary DNA: structural and

594            functional kinship with the glucocorticoid receptor. *Science* **237**, 268 (1987).

595     7.     A. L. Albiston, V. R. Obeyesekere, R. E. Smith, Z. S. Krozowski, Cloning and tissue

596            distribution of the human 1 lβ-hydroxysteroid dehydrogenase type 2 enzyme. *Molecular*

597            *and Cellular Endocrinology* **105**, R11 (1994).

598    8.    R. G. Deeley, Transmembrane Transport of Endo- and Xenobiotics by Mammalian ATP-

599          Binding Cassette Multidrug Resistance Proteins. *Physiological Reviews* **86**, 849 (2006).

600    9.    O. C. Meijer, E. C. de Lange, D. D. Breimer, A. G. de Boer, J. O. Workel, E. R. de Kloet,

601          Penetration of dexamethasone into brain glucocorticoid targets is enhanced in mdr1A P-

602          glycoprotein knockout mice. *Endocrinology* **139**, 1789 (1998).

603    10.   A. M. Karssen, O. C. Meijer, I. C. J. van der Sandt, P. J. Lucassen, E. C. M. de Lange, A.

604          G. de Boer, E. R. de Kloet, Multidrug Resistance P-Glycoprotein Hampers the Access of

605          Cortisol But Not of Corticosterone to Mouse and Human Brain. *Endocrinology* **142**, 2686

606          (2001).

607    11.   J. I. Webster, J. Carlstedt-Duke, Involvement of multidrug resistance proteins (MDR) in the

608          modulation of glucocorticoid response. *The Journal of steroid biochemistry and molecular*

609          *biology* **82**, 277 (2002).

610    12.   B. L. Mason, C. M. Pariante, S. A. Thomas, A revised role for P-glycoprotein in the brain

611          distribution of dexamethasone, cortisol, and corticosterone in wild-type and ABCB1A/B-

612          deficient mice. *Endocrinology* **149**, 5244 (2008).

613    13.   P. J. Raubenheimer, E. A. Young, R. Andrew, J. R. Seckl, The role of corticosterone in

614          human hypothalamic? pituitary?adrenal axis feedback. *Clin Endocrinol* **65**, 22 (2006).

615    14.   M. Wabitsch, R. E. Brenner, I. Melzner, M. Braun, P. Möller, E. Heinze, K. M. Debatin, H.

616          Hauner, Characterization of a human preadipocyte cell strain with high capacity for adipose

617          differentiation. *Int J Obes Relat Metab Disord* **25**, 8 (2001).

618    15.   V. Gekeler, W. Ise, K. H. Sanders, W. R. Ulrich, J. Beck, The Leukotriene LTD4 Receptor

619          Antagonist Mk571 Specifically Modulates MRP Associated Multidrug Resistance.

620          *Biochemical and Biophysical Research Communications* **208**, 345 (1995).

621   16.   F. Tiberghien, F. Loor, Ranking of P-glycoprotein substrates and inhibitors by a calcein-

622         AM fluorometry screening assay. *Anti-Cancer Drugs* **7**, 568 (1996).

623   17.   K. J. McInnes, C. J. Kenyon, K. E. Chapman, D. E. Livingstone, L. J. Macdonald, B. R.

624         Walker, R. Andrew, 5alpha-reduced glucocorticoids, novel endogenous activators of the

625         glucocorticoid receptor. *J Biol Chem* **279**, 22908 (2004).

626   18.   D. A. Stavreva, M. Wiench, S. John, B. L. Conway-Campbell, M. A. McKenna, J. R.

627         Pooley, T. A. Johnson, T. C. Voss, S. L. Lightman, G. L. Hager, Ultradian hormone

628         stimulation induces glucocorticoid receptor-mediated pulses of gene transcription. *Nature*

629         *Cell Biology* **11**, 1093 (2009).

630   19.   T. E. Reddy, J. Gertz, G. E. Crawford, M. J. Garabedian, R. M. Myers, The Hypersensitive

631         Glucocorticoid Response Specifically Regulates Period 1 and Expression of Circadian

632         Genes. *Molecular and Cellular Biology* **32**, 3756 (2012).

633   20.   K. A. Hughes, R. M. Reynolds, R. Andrew, H. O. Critchley, B. R. Walker, Glucocorticoids

634         turn over slowly in human adipose tissue in vivo. *J Clin Endocrinol Metab* **95**, 4696

635         (2010).

636   21.   R. Andrew, K. Smith, G. C. Jones, B. R. Walker, Distinguishing the Activities of 11β-

637         Hydroxysteroid Dehydrogenases in Vivo Using Isotopically Labeled Cortisol. *J Clin*

638         *Endocrinol Metab* **87**, 277 (2002).

639   22.   D. P. Macfarlane, S. Forbes, B. R. Walker, Glucocorticoids and fatty acid metabolism in

640         humans: fuelling fat redistribution in the metabolic syndrome. *Journal of Endocrinology*

641         **197**, 189 (2008).

642   23.   M. L. Sweat, Adrenocorticosteroids in peripheral and adrenal venous blood of man. *J Clin*

643         *Endocrinol Metab* **15**, 1043 (1955).

644 24. R. E. Peterson, Plasma corticosterone and hydrocortisone levels in man. *J Clin Endocrinol*

645 *Metab* **17**, 1150 (1957).

646 25. R. S. Ely, E. R. Hughes, V. C. Kelley, Studies of adrenal corticosteroids. I. Estimation of

647 plasma corticosterone and cortisol. *J Clin Endocrinol Metab* **18**, 190 (1958).

648 26. R. Fraser, V. H. James, Double isotope assay of aldosterone, corticosterone and cortisol in

649 human peripheral plasma. *The Journal of endocrinology* **40**, 59 (1968).

650 27. K. J. Huther, H. R. Scholz, Plasma concentrations and rates of plasma clearance and

651 production of cortisol and corticosterone in healthy persons and in subjects with

652 asymptomatic and clinical diabetes mellitus. *Hormone and metabolic research = Hormon-*

653 *und Stoffwechselforschung = Hormones et metabolisme* **2**, 357 (1970).

654 28. R. G. Dluhy, L. Axelrod, R. H. Underwood, G. H. Williams, Studies of the control of

655 plasma aldosterone concentration in normal man. II. Effect of dietary potassium and acute

656 potassium infusion. *J Clin Invest* **51**, 1950 (1972).

657 29. H. H. Newsome, Jr., A. S. Clements, E. H. Borum, The simultaneous assay of cortisol,

658 corticosterone, 11-deoxycortisol, and cortisone in human plasma. *J Clin Endocrinol Metab*

659 **34**, 473 (1972).

660 30. C. J. Oddie, J. P. Coghlan, B. A. Scoggins, Plasma desoxycorticosterone levels in man with

661 simultaneous measurement of aldosterone, corticosterone, cortisol and 11-deoxycortisol. *J*

662 *Clin Endocrinol Metab* **34**, 1039 (1972).

663 31. C. D. West, D. K. Mahajan, V. J. Chavre, C. J. Nabors, F. H. Tyler, Simultaneous

664 measurement of multiple plasma steroids by radioimmunoassay demonstrating episodic

665 secretion. *J Clin Endocrinol Metab* **36**, 1230 (1973).

666    32.    S. Nishida, S. Matsumura, M. Horino, H. Oyama, A. Tenku, The Variations of Plasma

667           Corticosterone/Cortisol Ratios Following ACTH Stimulation or Dexamethasone

668           Administration in Normal Men. *The Journal of Clinical Endocrinology & Metabolism* **45**,

669           585 (1977).

670    33.    J. Wijnholds, R. Evers, M. R. van Leusden, C. A. A. M. Mol, G. J. R. Zaman, U. Mayer, J.

671           H. Beijnen, M. V. D. Valk, P. Krimpenfort, P. Borst, Increased sensitivity to anticancer

672           drugs and decreased inflammatory response in mice lacking the multidrug resistance-

673           associated protein. *Nature Medicine* **3**, 1275 (1997).

674    34.    S. Dinneen, A. Alzaid, J. Miles, R. Rizza, Metabolic effects of the nocturnal rise in cortisol

675           on carbohydrate metabolism in normal humans. *J Clin Invest* **92**, 2283 (1993).

676    35.    C. B. Djurhuus, C. H. Gravholt, S. Nielsen, A. Mengel, J. S. Christiansen, O. E. Schmitz,

677           N. Moller, Effects of cortisol on lipolysis and regional interstitial glycerol levels in

678           humans. *American journal of physiology. Endocrinology and metabolism* **283**, E172

679           (2002).

680    36.    D. J. Wake, R. H. Stimson, G. D. Tan, N. Z. M. Homer, R. Andrew, F. Karpe, B. R.

681           Walker, Effects of Peroxisome Proliferator-Activated Receptor-α and -γ Agonists on 11β-

682           Hydroxysteroid Dehydrogenase Type 1 in Subcutaneous Adipose Tissue in Men. *The*

683           *Journal of Clinical Endocrinology & Metabolism* **92**, 1848 (2007).

684    37.    K. J. McInnes, L. B. Smith, N. I. Hunger, P. T. K. Saunders, R. Andrew, B. R. Walker,

685           Deletion of the Androgen Receptor in Adipose Tissue in Male Mice Elevates Retinol

686           Binding Protein 4 and Reveals Independent Effects on Visceral Fat Mass and on Glucose

687           Homeostasis. *Diabetes* **61**, 1072 (2012).

688    38.    V. van Harmelen, T. Skurk, H. Hauner, Primary culture and differentiation of human

689            adipocyte precursor cells. *Methods in molecular medicine* **107**, 125 (2005).

690    39.    R. Mouras, P. Bagnaninchi, A. Downes, A. Elfick, Multimodal, label-free nonlinear optical

691            imaging for applications in biology and biomedical science. *Journal of Raman*

692            *Spectroscopy* **44**, 1373 (2013).

693    40.    K. J. McInnes, C. J. Kenyon, K. E. Chapman, D. E. W. Livingstone, L. J. Macdonald, B. R.

694            Walker, R. Andrew, 5 -Reduced Glucocorticoids, Novel Endogenous Activators of the

695            Glucocorticoid Receptor. *Journal of Biological Chemistry* **279**, 22908 (2004).

696    41.    M. W. Voice, J. R. Seckl, K. E. Chapman, The sequence of 5′ flanking DNA from the

697            mouse 11 β-hydroxysteroid dehydrogenase type 1 gene and analysis of putative

698            transcription factor binding sites. *Gene* **181**, 233 (1996).

699    42.    T. E. R. Baudoux, A. A. Pozdzik, V. M. Arlt, E. G. De Prez, M.-H. Antoine, N. Quellard,

700            J.-M. Goujon, J. L. Nortier, Probenecid prevents acute tubular necrosis in a mouse model

701            of aristolochic acid nephropathy. *Kidney Int* **82**, 1105 (2012).

702    43.    T. Reinard, H.-J. Jacobsen, An inexpensive small volume equilibrium dialysis system for

703            protein-ligand binding assays. *Analytical Biochemistry* **176**, 157 (1989).

704

724    **Figures:**

725    **Fig. 1**. *ABCC1*, but not *ABCB1*, is expressed in human adipose tissue. **(A)** Transcript microarray

726    data for members of the ABC transporter family, ranked by intensity, in subcutaneous adipose

727    tissue from 12 healthy men. *ABCC1* but not *ABCB1* is highly expressed in human adipose.

728    Reverse transcriptase polymerase chain reaction amplification (RT-PCR) showing expression of

729    *ABCB1* and *ABCC1* in **(B)** human tissues and fully differentiated SGBS human adipocytes, **(C)**

730    murine adipose, and **(D)** horse adipose. MNC = mononuclear cells, Sk. Mus = skeletal muscle.

731

732    **Fig. 2.** ABCC1 preferentially exports corticosterone from adipocytes. **(A)** Export of $^{3}$H-cortisol

733    and $^{3}$H-corticosterone from SGBS adipocytes after steroid loading for the previous 24 hours (480

734    nM unlabeled steroid + 20 nM tritiated steroid). Data are expressed as a percentage of

735    intracellular steroid counts per minute (CPM) after 24 hour loading ($n = 3$, *$P<0.05$, **$P<0.01$,

736    two-way ANOVA with Bonferroni post-hoc test). **(B)** Coherent anti-Stokes Raman Scattering

737    (CARS) images of SGBS cells treated with D8-corticosterone (500 nM) with or without ABCC1

738    inhibition (MK571, 10 µM, 24 hours). Resonance of the C-H bonds abundant in lipid is

739    represented in red; C-D resonance from D8-corticosterone is represented in green, and is more

740    abundant with MK571 inhibition, notably in a distribution around the intracellular lipid droplets

741    (scale bar = 20 µm). **(C)** Effect of ABCC1 inhibition with either MK571 (10 µM) or probenecid

742    (PBN, 50 µM) on $^{3}$H-cortisol and $^{3}$H-corticosterone retention in SGBS adipocytes after 24 hour

743    incubation. ($n = 3$, ***$P<0.001$ vs Vehicle, ##$P<0.01$, ###$P<0.001$ vs cortisol, two-way

744    ANOVA with Bonferroni post-hoc test). **(D)** SGBS adipocytes incubated with corticosterone or

745    cortisol (500 nM, 2 hours) in the presence or absence of probenecid (PBN, 50 µM). Transcripts

746    measured are period circadian clock (*PER1*), adiponectin (*ADIPOQ*), adipose triglyceride lipase

747    (*ATGL*), and hormone sensitive lipase (*HSL*). (*n* = 3, *$P<0.05$ vs Control, #$P<0.05$ vs – PBN,

748    two-way ANOVA with Bonferroni post-hoc test. **(E)** SGBS pre-adipocytes show greater

749    triglyceride accumulation after incubation with cortisol than corticosterone (100 nM), quantified

750    by Oil Red O staining (*n* = 3, ***$P<0.001$ vs corticosterone, two-way ANOVA with Bonferroni

751    post-hoc test). All data are mean ± SEM, exact *P* values are given in table S7.

752

753    **Fig. 3**. *Abcc1$^{-/-}$* deletion or pharmacological inhibition in mice results in preferential

754    accumulation of corticosterone in adipose tissue. **(A - C)** Adrenalectomized male wild type

755    (*Abcc1$^{+/+}$*) or ABCC1 knockout (*Abcc1 $^{-/-}$*) FVB mice were infused with corticosterone and

756    cortisol for 7 days. **(D - E)** Adrenalectomized male C57Bl/6 mice were infused with

757    corticosterone and cortisol, together with either probenecid or vehicle (saline), for 7 days. Plasma

758    (A,D), brain (B,E), and subcutaneous adipose (C,F) corticosterone (C'one) and cortisol were

759    quantified by LC/MS-MS and are presented as concentrations and as corticosterone:cortisol

760    ratios (Ratio). All data are mean ± SEM, *n* = 6-8 per group, (A - C) *$P<0.05$ vs *Abcc1$^{+/+}$*, (D - E)

761    *$P <0.05$ vs vehicle, Student's t test, exact *P* values are given in table S7.

762

763    **Fig. 4.**  Pharmacological inhibition of ABCC1 potentiates adipose GC-responsive transcript

764    expression. **(A)** Plasma corticosterone (C'one) and cortisol concentrations, and their ratio, were

765    not altered between mice receiving 7 days treatment with corticosteroid (corticosterone and

766    cortisol 250 µg/day) in the presence or absence of probenecid (150 mg/kg/day).

767    Adrenalectomized mice not receiving corticosteroid infusion had corticosterone or cortisol

768    concentrations below the detectable limit (0.5 ng/ml). **(B)** Change in body weight of

769    adrenalectomized mice after 7 days of treatment with corticosteroid (steroid) in the presence or

770    absence of probenecid (PBN), or control (no corticosteroid). **(C)** Subcutaneous adipose transcript

771    expression of the glucocorticoid-responsive genes period circadian clock 1 (*Per1*), adipose

772    triglyceride lipase (*Atgl*), hormone sensitive lipase (*Hsl*), fatty acid synthase *(Fas)*,

773    diacylglycerol O-Acyltransferase 1 *(Dgat1),* and lipoprotein lipase *(Lpl).* All data are mean ±

774    SEM (*n* = 7-11 per group, *$P<0.05$ vs control, #$P<0.05$ vs steroid + vehicle, two-way ANOVA

775    with Bonferroni post-hoc test), exact *P* values are given in table S7.

776

777    **Fig. 5**. Corticosterone concentrations are low in human adipose tissue. Adipose biopsies were

778    obtained during elective abdominal surgery from 6 lean and 6 obese patients. **(A)** *ABCC1* mRNA

779    concentrations were upregulated in obese subcutaneous (SC) and visceral (Visc) adipose tissue.

780    (*n* = 6, *$P <0.05$, **$P<0.01$ vs lean, unpaired Student's t tests). **(B)** Adipose tissue cortisol

781    concentrations were readily detectable, but corticosterone concentrations were low or below the

782    limit of detection (LOD) in SC and visceral adipose [samples below limit of detection are

783    assigned a value of 0.86 pmoles/g (LOD)]. Neither cortisol nor corticosterone differed between

784    lean and obese participants. All data are mean ± SEM, exact *P* values are given in table S7.

785

786    **Fig. 6**. Human adipose tissue is more sensitive to cortisol than corticosterone. A randomized

787    single blind crossover study with ramped infusion of deuterated cortisol (D4-Cortisol) or

788    deuterated corticosterone (D8-Corticosterone) was conducted in 9 patients with Addison's

789    disease. Plasma concentrations of total cortisol and corticosterone **(A)** or ACTH **(B)** were not

790    significantly different throughout the study. **(C)** Suppression of ACTH was similar during high-

791    dose infusion of cortisol or corticosterone. **(D)** Cortisol induced a greater rise in *PER1* and *LPL*

792    mRNA concentrations in subcutaneous adipose tissue (*$P$<0.05 vs corticosterone, paired

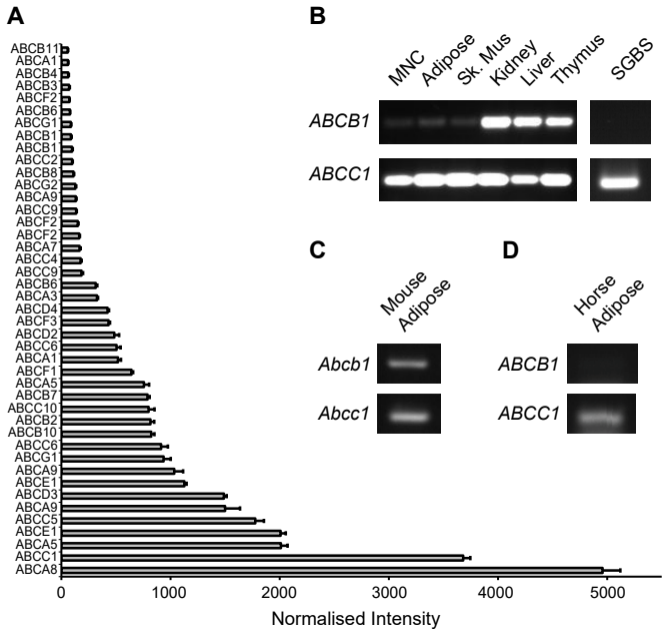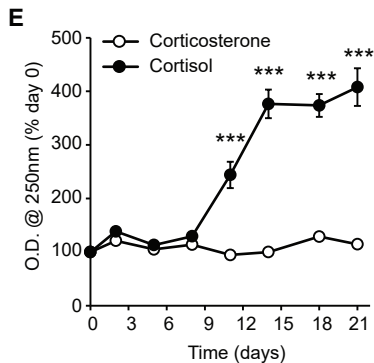793    Student's t-tests). All data are mean ± SEM, n=9, exact $P$ values are given in table S7.
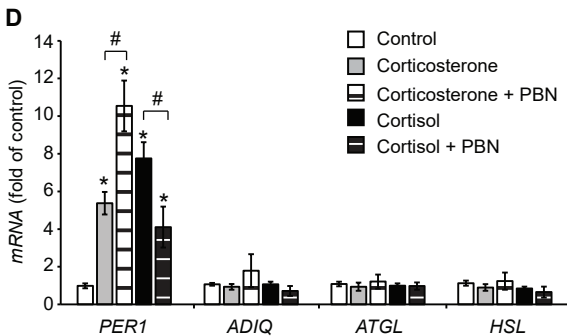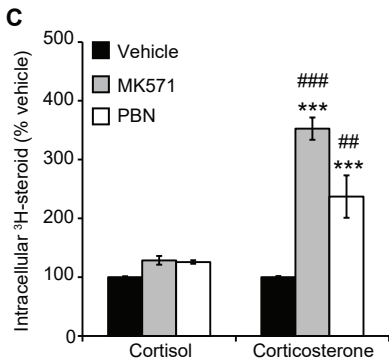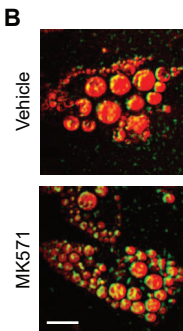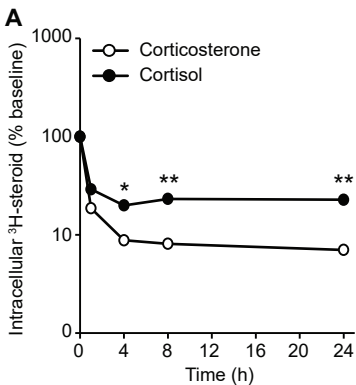
794

795    **Fig. 7.** Differential effects of cortisol and corticosterone in brain versus adipose are conferred by

796    tissue-specific expression of ABC transporters. In conventional glucocorticoid replacement

797    therapy, cortisol (hydrocortisone) action in the brain is limited by export through ABCB1, but

798    the absence of ABCB1 in human adipose tissue allows potent effects of cortisol on peripheral

799    metabolism. With corticosterone therapy, the lack of transport by ABCB1 in the brain allows

800    corticosterone to exert a potent effect to suppress ACTH, but in adipose tissue, corticosterone

801    action is limited by ABCC1, protecting against adverse effects of corticosterone on peripheral
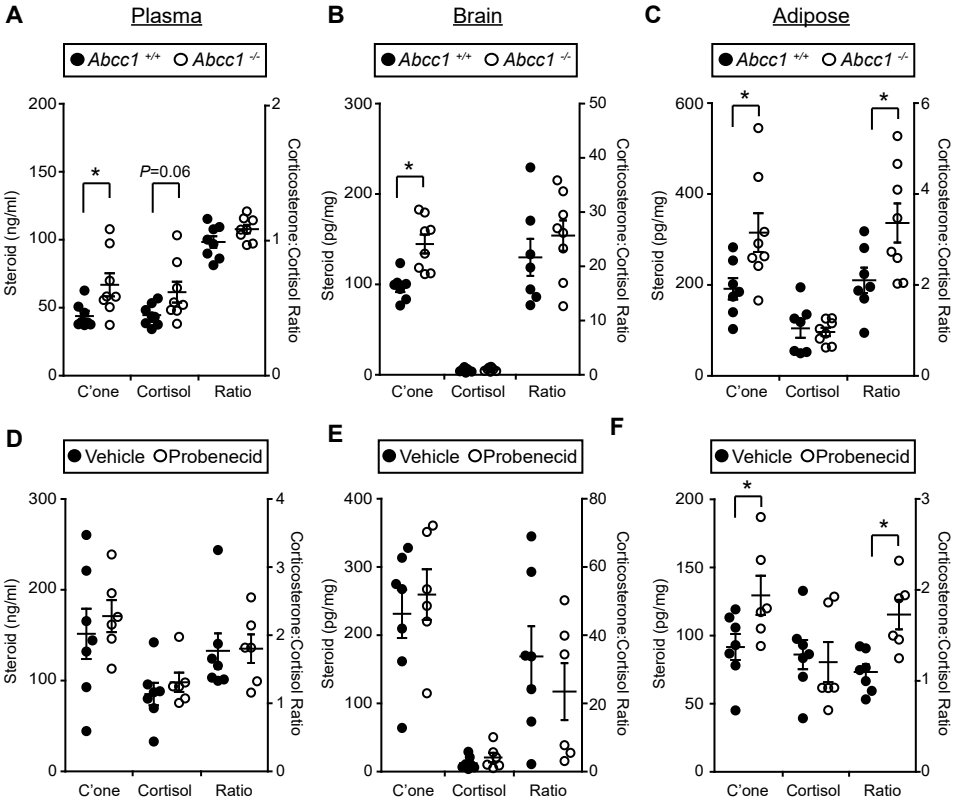
802    metabolism.

803  **Table 1.** Plasma biochemistry during infusion of either deuterated cortisol or corticosterone in patients with Addison's disease. Data

804  are mean ± SEM of within-subject averages from samples obtained during each steady state infusion period. $N = 9$. Comparisons were

805  by two-way repeated measures ANOVA: only ACTH ($P<0.001$), non-esterified fatty acids (NEFA, ($P=0.018$), and insulin ($P=0.042$)

806  changed with duration of steroid infusion, but there were no differences between corticosterone or cortisol infusions and no significant

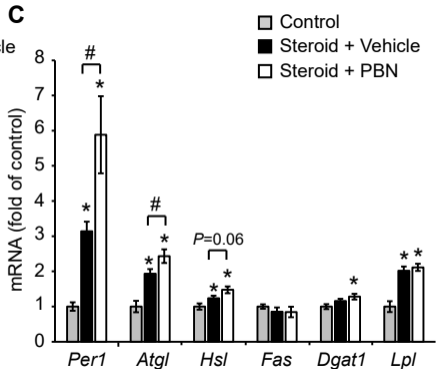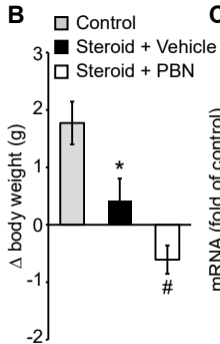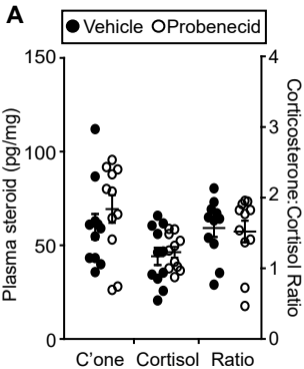807  interactions between steroid and duration.

| | Cortisol | | | | Corticosterone | | | |
|---|---|---|---|---|---|---|---|---|
| **Duration of infusion (min)** | *0-60* | *80-140* | *160-240* | *260-330* | *0-60* | *80-140* | *160-240* | *260-330* |
| Total deuterated steroid (nM) | 0 | 53 ± 11 | 190 ± 37 | 419 ± 74 | 0 | 34 ± 4 | 156 ± 15 | 340 ± 26 |
| Endogenous cortisol (nM) | 18 ± 4 | 12 ± 2 | 8 ± 2 | 5 ± 1 | 22 ± 7 | 16 ± 5 | 11 ± 3 | 7 ± 2 |
| ACTH (pM) | 42 ± 5 | 40 ± 5 | 37 ± 5 | 30 ± 5 | 42 ± 4 | 43 ± 4 | 38 ± 5 | 33 ± 5 |
| Glucose (mM) | 4.5 ± 0.1 | 4.3 ± 0.1 | 4.4 ± 0.2 | 4.6 ± 0.2 | 4.7 ± 0.2 | 4.8 ± 0.2 | 4.7 ± 0.2 | 4.8 ± 0.2 |
| Insulin (pM) | 44 ± 11 | 39 ± 7 | 38 ± 7 | 35 ± 6 | 43 ± 8 | 39 ± 6 | 37 ± 6 | 33 ± 5 |
| NEFA (µM) | 333 ± 54 | 415 ± 59 | 474 ± 63 | 477 ± 60 | 338 ± 47 | 391 ± 50 | 420 ± 66 | 412 ± 58 |

808

**A**

Intracellular $^3$H-steroid (% baseline)

○ Corticosterone
● Cortisol

* ** **

Time (h)

**B**

Vehicle

MK571

**C**

Intracellular $^3$H-steroid (% vehicle)

■ Vehicle
▨ MK571
□ PBN

### ###
***
##
***

Cortisol    Corticosterone

**D**

mRNA (fold of control)

□ Control
▨ Corticosterone
▤ Corticosterone + PBN
■ Cortisol
▬ Cortisol + PBN

#
*
*
*
#
*
*

PER1    ADIQ    ATGL    HSL

**E**

O.D. @ 250nm (% day 0)

○ Corticosterone
● Cortisol

***  ***  ***  ***

Time (days)

**A** Plasma
● *Abcc1* [+/+]  ○ *Abcc1* [-/-]

**B** Brain
● *Abcc1* [+/+]  ○ *Abcc1* [-/-]

**C** Adipose
● *Abcc1* [+/+]  ○ *Abcc1* [-/-]

**D** ● Vehicle ○ Probenecid

**E** ● Vehicle ○ Probenecid

**F** ● Vehicle ○ Probenecid

**A**

Plasma steroid (pg/mg) / Corticosterone:Cortisol Ratio

● Vehicle ○ Probenecid

C'one   Cortisol   Ratio

**B**

Δ body weight (g)

Control
Steroid + Vehicle
Steroid + PBN

**C**

mRNA (fold of control)

Control
Steroid + Vehicle
Steroid + PBN

*Per1*   *Atgl*   *Hsl*   *Fas*   *Dgat1*   *Lpl*

*P*=0.06

**A**

Adipose *ABCC1* mRNA (A.U.)

△ Lean
▲ Obese

SC Adipose   Visc Adipose

**B**

Adipose steroid conc (pmoles/g)

△ Lean
▲ Obese

C'one   Cortisol   C'one   Cortisol
SC Adipose          Visc Adipose

**A**

Steroid (nM)

○ Corticosterone
● Cortisol

Time (min)

**B**

ACTH (pM)

○ Corticosterone
● Cortisol

Time (min)

**C**

ACTH (% baseline)

C'one    Cortisol

**D**

mRNA/18S (A.U.)

○ Corticosterone
● Cortisol

*    *

PER1    ADIQ    ATGL    HSL    LPL

| Brain | Adipose |
|---|---|

**Cortisol replacement**

ABCB1

*Cortisol export*

No cortisol export

ABCC1

GR

**Reduced cortisol response**

GR

Cortisol response
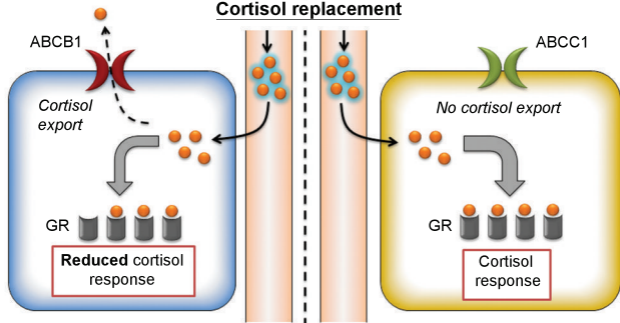
**Corticosterone replacement**

ABCB1

*No corticosterone export*

*Corticosterone export*

ABCC1

GR

Corticosterone response
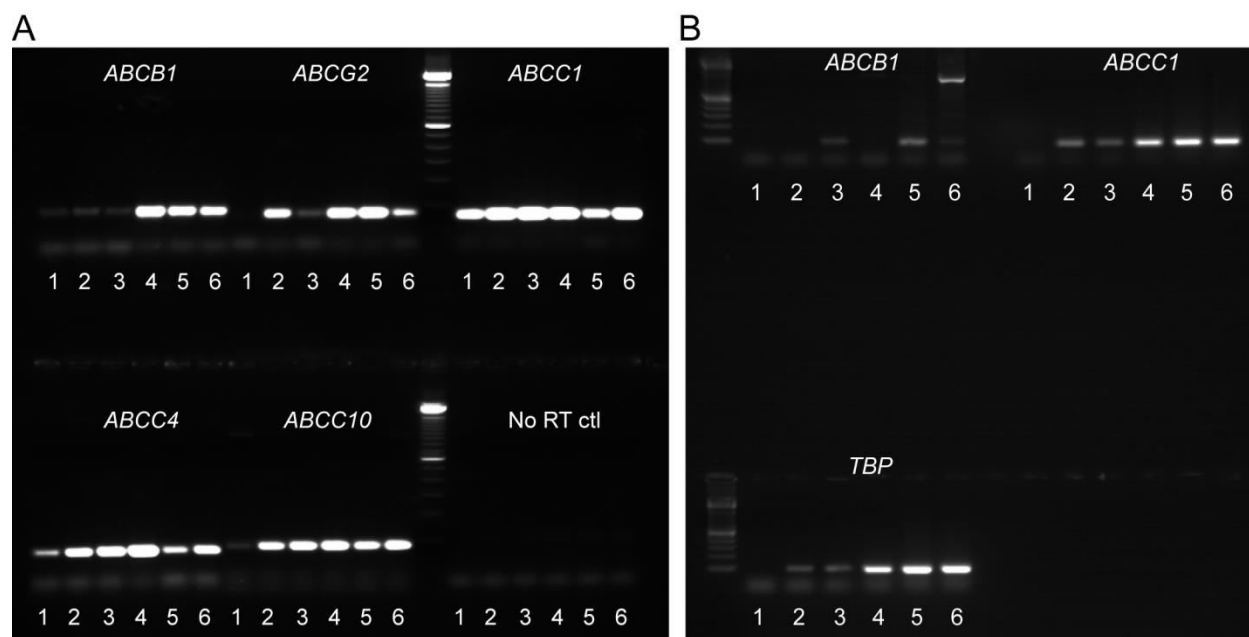
GR

**Reduced corticosterone response**
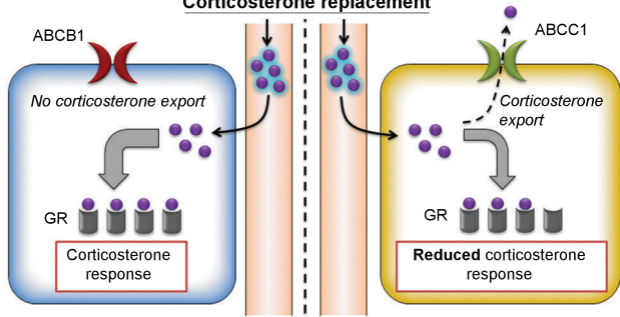
Key: ● Cortisol ● Corticosterone ◖◗ ABCB1 ◖◗ ABCC1 ▬ GR

1    **Supplementary Materials:**



2

3    **Fig. S1.** Whole gel PCR image of ABC transporter expression in tissues and cells. **(A)** PCR gel

4    of ABC transporter expression from human tissues. Lanes are denoted as follows: 1 =

5    mononuclear cells, 2 = adipose, 3 = skeletal muscle, 4 = kidney, 5 = liver, 6 = thymus. Genes are

6    indicated above bands. No RT ctl = negative control (without reverse transcriptase enzyme) for

7    each sample. **(B)** Whole gel PCR image of *ABCB1, ABCC1,* and *TBP* in various cell lines. Lanes

8    are denoted as follows:  1 = negative control, 2 = A549, 3 = HepG2, 4 = SGBS, 5 = HeLa, 6 =

9    MCF-7.

10



11

12
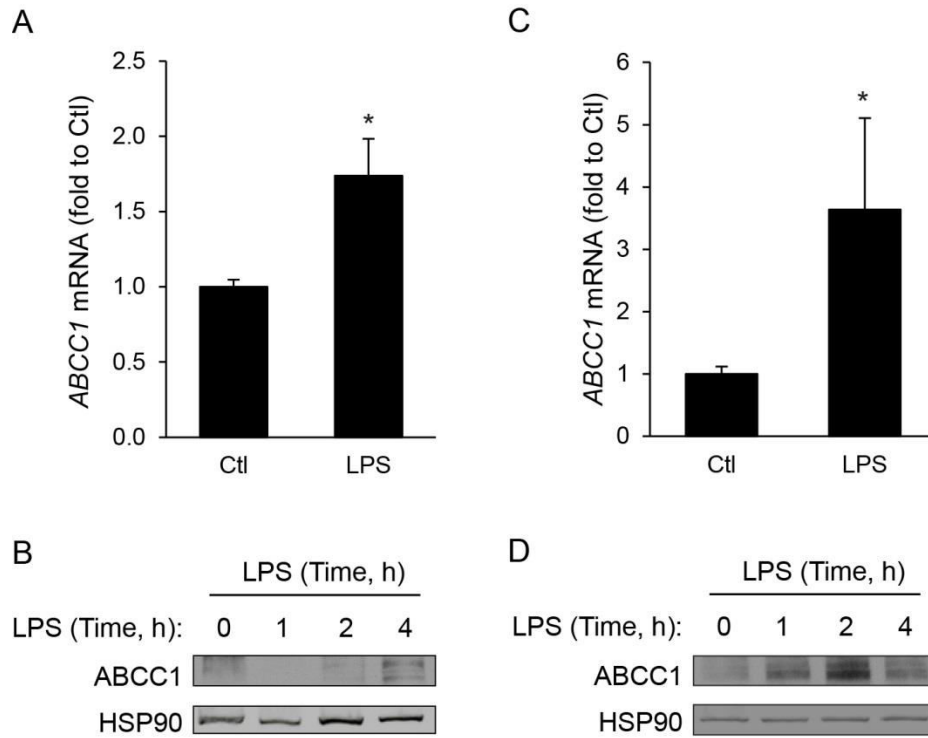
**Fig. S2.** Correlation of *ABCC1* mRNA and protein concentrations in human adipocytes. (**A** and

**B**) SGBS adipocytes and (**C** and **D**) primary human adipocytes treated with lipopolysaccharide

(100 ng/ml) for the indicated times. mRNA concentrations after 2 hours of treatment (**A** and **C**)

correlate with protein concentrations (**B** and **D**). Data for mRNA are mean ± SEM, $n = 3$, *

*P*<0.05 vs control (Ctl).
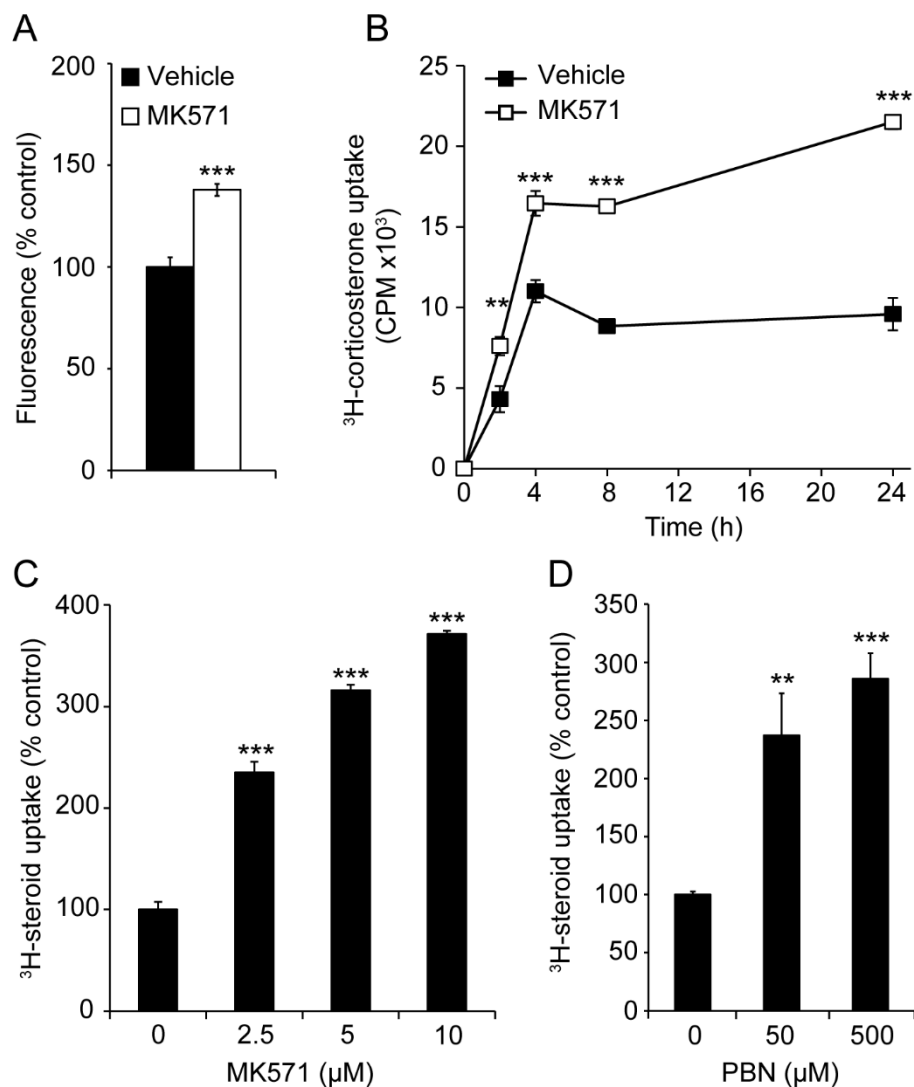
18

19   **Fig. S3.** ABCC1 inhibition in SGBS adipocytes. **(A)** SGBS adipocytes incubated with Calcein-

20   AM for 90 min demonstrate the extent of ABCC1 inhibition by MK571 (10 µM; $n = 6$, Student's

21   t-test: ***$P<0.001$ vs control). **(B)** Time course of ^{3}H-corticosterone accumulation with ABCC1

22   inhibition (MK571, 10 µM; $n = 3$, two-way ANOVA with Bonferroni post-hoc test: **$P<0.01$,

23   ***$P<0.001$). Dose response of ^{3}H-corticosterone accumulation over 24 hours after ABCC1

24      inhibition with various concentrations of **(C)** MK571, or **(D)** probenecid (PBN; $n = 3$, one-way

25      ANOVA: **$P<0.01$, ***$P<0.001$ vs 0). All data are mean ± SEM.

26
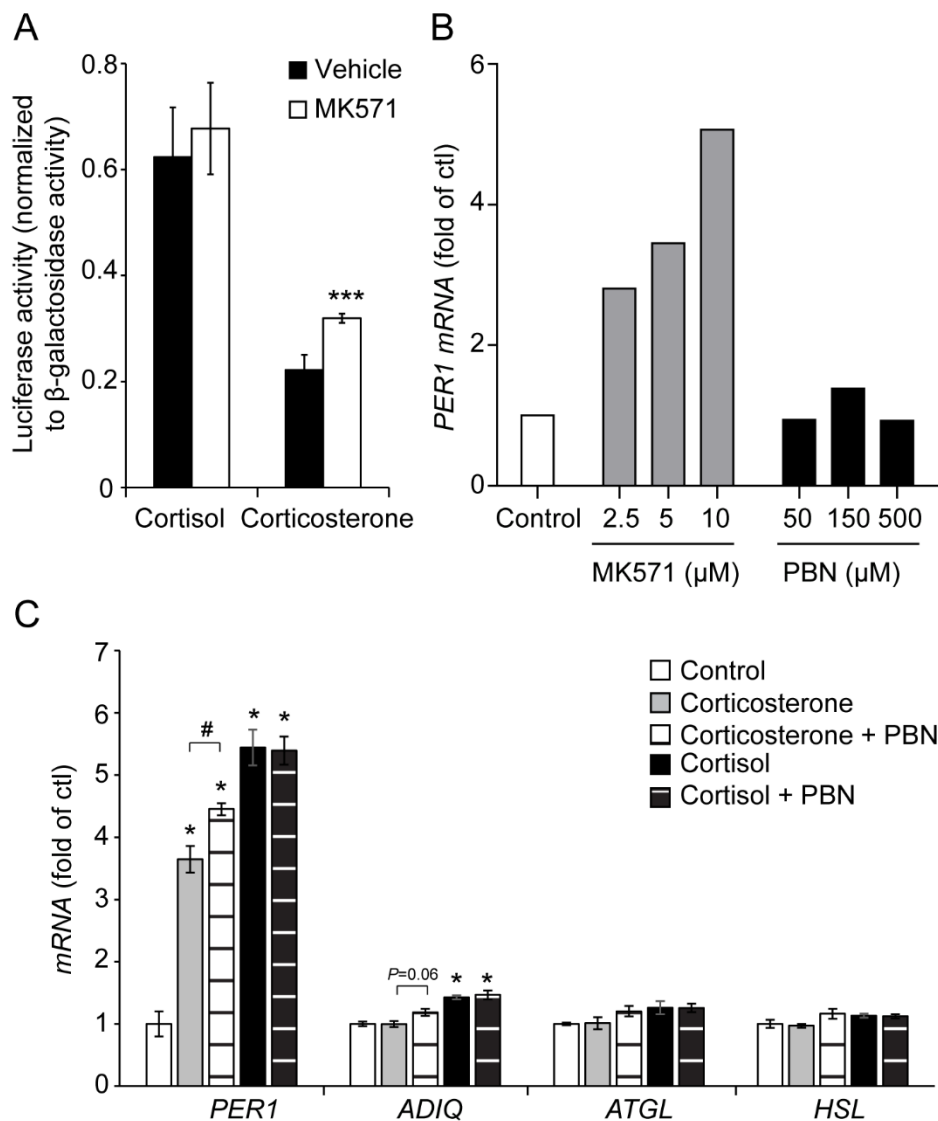
**Fig. S4.** Effects of ABCC1 inhibition on GR-mediated transcription. **(A)** A549 cells transfected

with glucocorticoid-responsive MMTV-luciferase reporter and treated with corticosterone or

cortisol (500 nM, 24 hours) show potentiation of response to corticosterone but not cortisol by

ABCC1 inhibition (MK571, 10 µM; $n = 3$, two-way ANOVA with Bonferroni post-hoc test:

***$P<0.001$ vs vehicle). **(B)** Glucocorticoid-responsive period circadian clock 1 (*PER1*) gene

expression in SGBS adipocytes treated with MK571 or probenecid (PBN) for 24 hours, $n = 2$.

33    **(C)** Glucocorticoid-responsive gene expression in SGBS adipocytes treated with corticosterone

34    or cortisol (500 nM) in the presence of absence of probenecid (PBN, 50 µM) for 24 hours.

35    Glucocorticoid-responsive genes are *PER1*, adiponectin (*ADIQ*), adipose triglyceride lipase

36    (*ATGL*), and hormone sensitive lipase (*HSL*), ($n$ = 3, Student's t-test: \*$P<0.05$ vs control;

37    #$P<0.05$ vs PBN). All data are mean ± SEM.

38

39
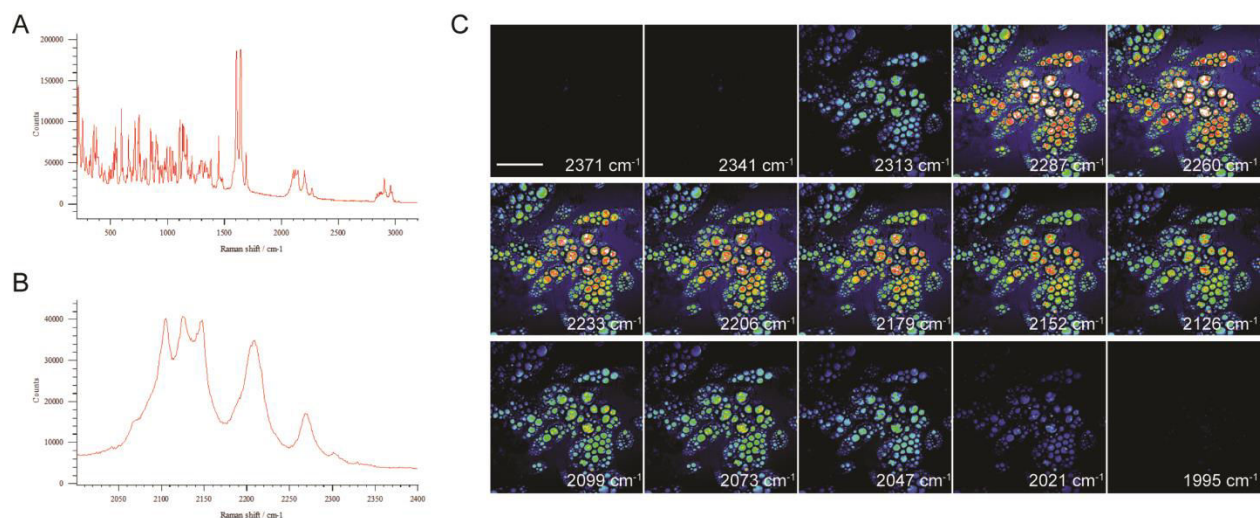
**Fig. S5.** Optimization of CARS microscopy for detection of intracellular D8-corticosterone. **(A)** Raman spectra of D8-corticosterone showing peaks representing C-D bonds between 2000 and 2300 cm$^{-1}$. **(B)** Magnified Raman spectrum of D8-corticosterone between 2000 and 2400 cm$^{-1}$. **(C)** Coherent Anti-stokes Raman Scattering (CARS) images of SGBS adipocytes treated with D8-corticosterone (10 µM, 24 hours) probed at inverse wavenumbers corresponding to the Raman spectrum and showing optimal detection at 2126 cm$^{-1}$ (scale bar = 100 µm).

46      **Table S1.** Characteristics of lean and obese study participants providing adipose biopsy samples

47      during surgery.

48

|  | Lean | Obese |
|---|---|---|
| Age (years) | $60 \pm 4$ | $59 \pm 3$ |
| Gender (M/F) | 2/4 | 2/4 |
| Body mass index (kg/m$^2$) | $23.9 \pm 0.3$ | $41.4 \pm 1.9$ |

49

50     **Table S2.** Characteristics of Addison's study participants.

51

|  | Mean ± SEM | Range |
|---|---|---|
| Age (years) | 53 ± 5 | 20 - 65 |
| Gender (M/F) | 3/6 | |
| Body mass index (kg/m$^2$) | 24.7 ± 1.2 | 21.7 - 32.9 |
| Years since diagnosis | 19 ± 3 | 4 - 30 |
| Daily hydrocortisone dose (mg) | 21 ± 2 | 15 - 30 |
| Daily fludrocortisone dose (µg) | 76 ± 30 | 0 - 300 |

52

53

54    **Table S3.** Summary of studies describing plasma corticosterone and cortisol concentrations in healthy subjects.

| Reference | Gender (M/F) | Age (years) | Corticosterone (B) nM | Cortisol (F) nM | B/F[‡] | n | Time | B assay Technique |
|---|---|---|---|---|---|---|---|---|
| Sweat, 1955 (23) | ns | ns | 124.1 ± 14.4 | 298.0 ± 16.6 | 0.416 | 21 | ns | Fluorometric method |
| Peterson, 1957 (24) | 18/12 | ns | 31.7 ± 11.5 | 386.2 ± 33.1 | 0.082 | 30 | ns | Isotope dilution/fluorescence |
| Ely et al, 1958 (25) | ns | ns | 86.6 ± 5.8 | 300.7 ± 15.1 | 0.288 | 20 | ns | Fluorometric method |
| Fraser et al, 1968 (26) | ns | ns | 19.0 (3.8-66.4) | 270.3 (85.5-557) | 0.067 | 29 | ns | Double isotope assay |
| Huther et al, 1970 (27) | M<br>F | 26.8 ± 2.6<br>31.3 ± 4.4 | 47.2 ± 6.0<br>49.7 ± 6.5 | 403.0 ± 37.5<br>422.4 ± 67.8 | 0.124<br>0.118 | 9[†]<br>10[†] | 1045-1145[*] | Fluorometric method |
| Dluhy et al, 1972 (28) | 8/2 | 21-34 | 26.3 ± 3.2 | 634.5 ± 55.2 | 0.041 | 10 | 0900 | Radioimmunoassay |
| Newsome, Jr. et al 1972 (29) | ns | ns | 11.5 ± 0.9 | 339.3 ± 22.1 | 0.034 | 8 | ns | Competitive protein binding |
| Oddie et al, 1972 (30) | ns | ns | 12.1 ± 2.6 | 383.4 ± 4.0 | 0.030 | 18 | 0900 | Double isotope assay |
| West et al, 1973 (31) | M<br>F[1]<br>F[2] | 19-50 | 11.4 ± 1.7<br>20.4 ± 2.0<br>16.7 ± 2.1 | 386 ± 36<br>386 ± 36<br>359 ± 21 | 0.030<br>0.053<br>0.047 | 15<br>9<br>9 | 0800 | Radioimmunoassay |
| Nishida et al, 1977 (32) | ns | ns | 23.0 ± 3.0 | 419.6 ± 28.7 | 0.055 | 10 | 0900 | Radioimmunoassay |
| Karssen et al, 2001 (10) | M | 57 ± 1.9 | 16.4 ± 3.1 | 350.0 ± 81.5 | 0.047 | 11 | ns | LC-MS |
| Raubenheimer et al, 2006 (13) | M | 39.2 (23-70) | 58.4 ± 9.2 | 830.4 ± 68.4 | 0.069 | 16 | 0830-0900 | Radioimmunoassay |

55
56    Mean ± SEM or range (). ns = not specified
57    [†] = mean of 5 samples for each subject
58    [*] = approximate time inferred from clinical protocol
59    [1] follicular phase, [2] luteal phase
60    [‡] mean [B] (nM)/mean [F] (nM)

61    **Table S4.** Primer sequences for PCR and corresponding expected product size.

| Gene ID | Primer sequence 5' to 3' | Expected product size (bp) |
|---|---|---|
| *ABCC1* Human | F: gcctattacccccagcatcg | 69 |
| | R: gatgcagttgcccacaca | |
| *ABCB1* Human | F: aaggcatttacttcaaacttgtca | 78 |
| | R: tggattcatcagctgcatttt | |
| *ABCG2* Human | F: tggcttagactcaagcacagc | 67 |
| | R: tcgtccctgcttagacatcc | |
| *ABCC4* Human | F: cctggcgaattgttagctgt | 68 |
| | R: agcacggcacttaacagtga | |
| *ABCC10* Human | F: agctcactgccaccaagg | 76 |
| | R: caagggaagttgttgagagga | |
| *Abcc1* Mouse | F: ggaattttcggctgagtgtc | 63 |
| | R: agccaaatattgctgcacct | |
| *Abcb1* Mouse | F: tgctttgtgggcaaaggta | 106 |
| | R: cacagttctgatggctgctaa | |
| *ABCB1* Horse | F: tcaggtggccctggataa | 157 |
| | R: cgaactgtagacaagcgatga | |
| *ABCC1* Horse | F: caaaatcatggctgccttaaa | 89 |
| | R: gaaagtgacatcgcgaaaca | |

62

63    **Table S5.** Human primer sequences for qPCR and corresponding probe number from Roche

64    Universal Probe Library (UPL).

| Gene ID | Primer sequence 5' to 3' | Roche UPL Probe Number |
|---|---|---|
| *ABCC1* Human | F: gcctattaccccagcatcg | 28 |
| | R: gatgcagttgcccacaca | |
| *ABCB1* Human | F: aaggcatttacttcaaacttgtca | 18 |
| | R: tggattcatcagctgcatttt | |
| *PER1* Human | F: ctcttccacagctccctca | 87 |
| | R: ctttggatcggcagtggt | |
| *LPL* Human | F: atgtggcccggtttatca | 25 |
| | R: ctgtatcccaagagatggacatt | |
| *ADIPONECTIN* Human | F: ggtgagaagggtgagaaagga | 85 |
| | R: tttcaccgatgtctcccttag | |
| *ATGL* Human | F: ctccaccaacatccacgag | 89 |
| | R: ccctgcttgcacatctctc | |
| *18S* Human | F: cttccacaggaggcctacac | 46 |
| | R: cgcaaaatatgctggaacttt | |

65   **Table S6.** Murine primer sequences for qPCR and corresponding probe number from Roche

66   Universal Probe Library (UPL).

| Gene ID | Primer sequence 5' to 3' | Roche UPL Probe Number |
|---|---|---|
| *Abcc1* Murine | F: ggaattttcggctgagtgtc | 105 |
| | R: agccaaatattgctgcacct | |
| *Abcb1* Murine | F: tgctttgtgggcaaaggta | 78 |
| | R: cacagttctgatggctgctaa | |
| *Per1* Murine | F: gcttcgtggacttgacacct | 71 |
| | R: tgctttagatcggcagtggt | |
| *Lpl* Murine | F: ctcgctctcagatgccctac | 95 |
| | R: ggttgtgttgcttgccatt | |
| *Atgl* Murine | F: gagcttcgcgtcaccaac | 89 |
| | R: cacatctctcggaggacca | |
| *Hsl* Murine | F: gcgctggaggagtgtttttt | 3 |
| | R: ccgctctccagttgaacc | |
| *Fas* Murine | F: ccaaatccaacatgggaca | 34 |
| | R: tgctccagggataacagca | |
| *Dgat1* Murine | F: gcttctgcagtttggagacc | 31 |
| | R: tccagttctgccaaaagtaggt | |
| *Tbp* Murine | F: gggagaatcatggaccagaa | 97 |
| | R: gatgggaattccaggagtca | |
| *18S* Murine | F: ctcaacacgggaaacctcac | 77 |
| | R: cgctccaccaactaagaacg | |

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