Identification of a human blood biomarker of pharmacological 11\(\beta\)-hydroxysteroid dehydrogenase 1 inhibition

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Abstract

Background and Purpose: 11\(\beta\)-hydroxysteroid dehydrogenase 1 (11\(\beta\)-HSD1) catalyzes the oxoreduction of cortisone to cortisol, thereby amplifying levels of active glucocorticoids. It is considered a pharmaceutical target in metabolic disease and cognitive impairments. 11\(\beta\)-HSD1 also converts some 7\(\alpha\)-oxo-steroids to their 7\(\beta\)-hydroxy forms. A recent study in mice described the ratio of tauroursodeoxycholic acid (TUDCA)/tauro-7\(\alpha\)oxolithocholic acid (T7\(\alpha\)oxoLCA) as a biomarker for decreased 11\(\beta\)-HSD1 oxoreductase activity. The present study aimed to evaluate the equivalent bile acid ratio glycoursodeoxycholic acid (GUDCA)/glyco-7\(\alpha\)oxolithocholic acid (G7\(\alpha\)oxoLCA) as a biomarker for pharmacological 11\(\beta\)-HSD1 inhibition in humans and compare it with the currently applied urinary (5\(\alpha\)-tetrahydrocortisol+tetrahydrocortisol)/tetrahydrocortisone ((5\(\alpha\)THF+THF)/THE) ratio. Experimental Approach: Bile acid profiles were analyzed by ultra-HPLC tandem-MS in blood samples from two independent, double-blind placebo-controlled clinical studies on the orally administered selective 11\(\beta\)-HSD1 inhibitor AZD4017. The blood GUDCA/G7\(\alpha\)oxoLCA ratio was compared with the urinary tetrahydro-glucocorticoid ratio for the ability to detect 11\(\beta\)-HSD1 inhibition. Key Results: No significant alterations were observed in the bile acid profiles following 11\(\beta\)-HSD1 inhibition by AZD4017, except for an increase of the secondary bile acid G7\(\alpha\)oxoLCA. The enzyme product/substrate ratio GUDCA/G7\(\alpha\)oxoLCA was found to be more reliable to detect 11\(\beta\)-HSD1 inhibition than the absolute G7\(\alpha\)oxoLCA concentration in both cohorts. Comparison of the blood GUDCA/G7\(\alpha\)oxoLCA ratio with the urinary (5\(\alpha\)THF+THF)/THE ratio revealed that both ratios successfully detect 11\(\beta\)-HSD1 inhibition. Conclusion and Implications: 11\(\beta\)-HSD1 inhibition does not cause major alterations in bile acid homeostasis. The GUDCA/G7\(\alpha\)oxoLCA ratio represents the first blood biomarker of pharmacological 11\(\beta\)-HSD1 inhibition and may replace or complement the urinary (5\(\alpha\)THF+THF)/THE ratio biomarker.
Identification of a human blood biomarker of pharmacological 11β-hydroxysteroid dehydrogenase 1 inhibition

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Running title: GUDCA/G7oxoLCA as biomarker of 11β-HSD1 activity

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0. **Abstract**

**Background and Purpose:** 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) catalyzes the oxoreduction of cortisone to cortisol, thereby amplifying levels of active glucocorticoids. It is considered a pharmaceutical target in metabolic disease and cognitive impairments. 11β-HSD1 also converts some 7oxo-steroids to their 7β-hydroxy forms. A recent study in mice described the ratio of tauroursodeoxycholic acid (TUDCA)/tauro-7oxolithocholic acid (T7oxoLCA) as a biomarker for decreased 11β-HSD1 oxoreductase activity. The present study aimed to evaluate the equivalent bile acid ratio glycoursodeoxycholic acid (GUDCA)/glyco-7oxolithocholic acid (G7oxoLCA) as a biomarker for pharmacological 11β-HSD1 inhibition in humans and compare it with the currently applied urinary ((5αTetrahydrocortisol+Tetrahydrocortisol)/Tetrahydrocortisone ((5αTHF+THF)/THE) ratio.

**Experimental Approach:** Bile acid profiles were analyzed by ultra-HPLC tandem-MS in blood samples from two independent, double-blind placebo-controlled clinical studies on the orally administered selective 11β-HSD1 inhibitor AZD4017. The blood GUDCA/G7oxoLCA ratio was compared with the urinary tetrahydro-glucocorticoid ratio for the ability to detect 11β-HSD1 inhibition.

**Key Results:** No significant alterations were observed in the bile acid profiles following 11β-HSD1 inhibition by AZD4017, except for an increase of the secondary bile acid G7oxoLCA. The enzyme product/substrate ratio GUDCA/G7oxoLCA was found to be more reliable to detect 11β-HSD1 inhibition than the absolute G7oxoLCA concentration in both cohorts. Comparison of the blood GUDCA/G7oxoLCA ratio with the urinary (5αTHF+THF)/THE ratio revealed that both ratios successfully detect 11β-HSD1 inhibition.

**Conclusion and Implications:** 11β-HSD1 inhibition does not cause major alterations in bile acid homeostasis. The GUDCA/G7oxoLCA ratio represents the first blood biomarker of pharmacological 11β-HSD1 inhibition and may replace or complement the urinary ((5αTHF+THF)/THE ratio biomarker.

**Keywords**

Bile acid; 11beta-hydroxysteroid dehydrogenase; biomarker; LC-MS; inhibitor; disease; glucocorticoid.

**Bullet point summary**

**What is already known**

- The glucocorticoid metabolizing enzyme 11β-HSD1 can also reduce the secondary bile acid 7-oxolithocholic acid.
- The TUDCA/T7oxoLCA ratio detects decreased 11β-HSD1 activity in transgenic mice and upon pharmacological inhibition.

**What this study adds**

- The blood GUDCA/G7oxoLCA ratio serves as biomarker of pharmacological 11β-HSD1 inhibition in humans.
- The blood GUDCA/G7oxoLCA ratio may substitute the currently used urinary tetrahydro-glucocorticoid biomarker.
- This study translates findings from preclinical investigations in mice to humans.

**Clinical significance**

- The blood GUDCA/G7oxoLCA ratio is a biomarker of pharmacological 11β-HSD1 inhibition in humans.
- It may replace or complement the currently used urinary tetrahydro-glucocorticoid biomarker.
- Plasma/serum samples are easier to handle than 24-hour urines, facilitating the evaluation of inhibitors.
1. **Introduction**

Glucocorticoids belong to the most widely prescribed drugs, used to treat diseases such as chronic asthma and different inflammatory conditions or as co-medication following organ transplantation (Cain & Cidlowski, 2017; Reichardt et al., 2021). However, the long-term pharmacological use of glucocorticoids or chronic excess of endogenous glucocorticoid levels promote various adverse health effects including cardio-metabolic complications, osteoporosis, glaucoma, skeletal muscle atrophy as well as depression and cognitive impairment. Glucocorticoids exert their functions mainly through activation of glucocorticoid receptors (GR). On a tissue- and cell-specific level, the concentrations of active glucocorticoids are tightly controlled by 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes (Gathercole et al., 2013; Odermatt & Kratschmar, 2012). 11β-HSD1 is widely expressed, and in metabolically active tissues such as liver, adipose, bone and skeletal muscle, it converts the inactive glucocorticoid cortisone to the active form cortisol (Figure 1). Inappropriately elevated levels of 11β-HSD1 activity have been implicated in many diseases including cardio-metabolic disorders, impaired wound healing and cognitive impairment; thus inhibition of 11β-HSD1 represents an attractive therapeutic strategy to lower glucocorticoid-mediated adverse effects (Gathercole et al., 2013; Gregory et al., 2020; Scott et al., 2014). For these reasons, a variety of pharmacological 11β-HSD1 inhibitors have been developed and tested in clinical trials (Bianzano et al., 2021; Courtney et al., 2008; Feig et al., 2011; Freude et al., 2016; Hardy et al., 2021; Heise et al., 2014; K. Markey et al., 2020; Othonos et al., 2023; Schwab et al., 2017; Shah et al., 2011; Webster et al., 2017).

Despite promising preclinical data, the translation to clinical application has been challenging. To better monitor pharmacological 11β-HSD1 inhibition in humans, the identification of novel non-invasive biomarkers reporting 11β-HSD1 inhibition could greatly facilitate optimization of treatment regimens. Current measurements to assess systemic 11β-HSD1 activity include the i.v. injection of prednisone followed by determination of the formed prednisolone in a blood sample (Bhat et al., 2008; Courtney et al., 2008), thus requiring an additional time-consuming and expensive intervention. Alternatively, systemic 11β-HSD1 activity in humans can be assessed by determining the ratio of the A-ring 5α- and 5β-reduced metabolites of cortisol and cortisone, *i.e.* (5α-tetrahydrocortisol (αTHF) + 5β-tetrahydrocortisol (THF))/tetrahydrocortisone (THE), and THF/THE in 24-hour urine samples (Bianzano et al., 2021; Courtney et al., 2008; Freude et al., 2016; Jamieson et al., 1999; K. Markey et al., 2020; Sagmeister et al., 2019; Tomlinson & Stewart, 2001; Webster et al., 2017). This ratio is also influenced by 11β-HSD2 that converts cortisol to cortisone, mainly in kidney and colon, exemplified by the highly elevated (αTHF+THF)/THE ratio in urines from patients with genetic defects in 11β-HSD2 (Odermatt et al., 2001; Palermo et al., 1996; Shackleton, 1993). Obtaining complete and accurate 24-hour urine samples is difficult, has poor patient acceptability and leads to challenges in sample storage and further processing. Currently, a suitable blood biomarker to report 11β-HSD1 inhibition is lacking.
11β-HSD1 is a multi-functional enzyme that in the presence of hexose-6-phosphate dehydrogenase (H6PD) predominantly functions as an oxoreductase (Odermatt & Klusonova, 2015). Earlier studies showed that human 11β-HSD1 is capable of metabolizing 7-oxo-cholesterol metabolites (Beck, Inderbinen, et al., 2019; Beck, Kanagaratnam, et al., 2019; Hult et al., 2004; Mitić et al., 2013; Schweizer et al., 2004) but also the secondary bile acid 7oxolithocholic acid (7oxoLCA) to ursodeoxycholic acid (UDCA) and to a lesser extent to chenodeoxycholic acid (CDCA) (Figure 1) (Mitić et al., 2013; Odermatt et al., 2011; Penno et al., 2013). A lack of this activity in guinea-pigs and in 11β-HSD1-deficient mice results in an accumulation of 7oxoLCA and its taurine conjugated form (Penno et al., 2013, 2014; Weingartner et al., 2021). A recent preclinical study employing three different transgenic mouse models of 11β-HSD1 deficiency and a model of pharmacological inhibition proposed the ratio of taurine conjugated UDCA (TUDCA) to 7oxoLCA (T7oxoLCA) in plasma samples as a biomarker to detect decreased 11β-HSD1 oxoreductase activity (Weingartner et al., 2021). The TUDCA/T7oxoLCA ratio was significantly lower in plasma from 11β-HSD1 knockout (KO) and H6PD KO mice and in plasma of mice treated with the inhibitor carbenoxolone.

The aim of the present study was to translate the observations made in these mouse models to humans and to identify an equivalent blood biomarker reporting a decreased 11β-HSD1 activity following pharmacological inhibition. In the mouse, bile acids are mainly conjugated with taurine but in humans with glycine and to a lesser extent with taurine, with an approximate ratio of 3:1 in human adult males (Russell & Setchell, 1992). Therefore, the ratio of glycine conjugated UDCA (GUDCA) to 7oxoLCA (G7oxoLCA) was tested. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was applied to assess the plasma or serum bile acid profiles in two different double-blind placebo-controlled clinical studies, a cohort of healthy males cotreated with prednisolone and a cohort of females with intracranial hypertension (Hardy et al., 2021; K. Markey et al., 2020; Othonos et al., 2023). Both groups were orally administered the selective 11β-HSD1 inhibitor AZD4017 (Scott et al., 2012). The effects of the inhibitor on the bile acid profile and the capability of the GUDCA/G7oxoLCA ratio to detect 11β-HSD1 inhibition were assessed.
**Figure 1.** Scheme of 11β-hydroxysteroid dehydrogenase (11β-HSD) activity. 11β-HSD1, in the presence of hexose-6-phosphate dehydrogenase (H6PD) catalyzes the oxoreduction of cortisone to cortisol and 7oxo-lithocholic acid (7oxoLCA) to ursodeoxycholic acid (UDCA) and lower amounts of chenodeoxycholic acid (CDCA). 11β-HSD2 catalyzes the oxidation of cortisol to cortisone but does not accept bile acids as substrates.
2. Materials and Methods

2.1. Chemicals and Reagents

Ultrapure water was obtained using a Milli-Q® Integral purification system equipped with an EDS-Pak® Endfilter for the removal of endocrine active substances (Merck Millipore, Burlington, MA, USA). Acetonitrile (HPLC-S Grade) was purchased from Biosolve (Dieuze, France), methanol (CHROMASOLV™ LC-MS grade) from Honeywell (Charlotte, NC, USA), isopropanol (EMSURE® for analysis) from Merck Millipore, and formic acid (Puriss. p.a. ≥ 98%) from Sigma-Aldrich (St. Louis, MO, USA). Bile acids and internal standards were purchased from Sigma-Aldrich or Steraloids (Newport, RI, USA) (Gómez et al., 2020). 11β-HSD1 inhibitor AZD4017 was obtained from AstraZeneca (Cambridge, UK) (Scott et al., 2012).

2.2. Clinical Cohorts

2.2.1. Cohort A

Healthy male volunteers without diabetes (n=29, BMI 20-30 kg/m², 18-60 years) were randomized in a double-blind placebo-controlled study to determine if co-administration of the selective 11β-HSD1 inhibitor AZD4017 limits the adverse effects of short-course exogenous glucocorticoid administration. All volunteers received 20 mg prednisolone once daily and 400 mg AZD4017 or placebo twice daily for 7 days. Blood samples were collected in the morning at baseline, and after 7 days of treatment, and serum was prepared and stored at -80°C until analysis. The trial was registered at Clinicaltrials.gov (NCT03111810, Targeting iatrogenic Cushing’s Syndrome with 11β-hydroxysteroid dehydrogenase Type 1 inhibition (TICSI)) (Othonos et al., 2023). The clinical parameters used for the calculation of correlations with the measured bile acids and ratios thereof are summarized in Supplemental Table S1.

2.2.2. Cohort B

Female patients (n=29, 18-55 years, BMI 25-52 kg/m²) with a clinical diagnosis of active idiopathic intracranial hypertension (IIH) (intracranial pressure (ICP) >25 cmH₂O and active papilledema) and normal brain imaging were randomized in a double-blind placebo-controlled study (K. Markey et al., 2020; K. A. Markey et al., 2017). Participants received twice daily for 12 weeks 400 mg of the oral selective 11β-HSD1 inhibitor AZD4017 or placebo. Morning blood samples were collected at baseline and following 12 weeks of treatment, and plasma was prepared by centrifugation at 4°C for 10 min at 1500 × g, aliquoted and stored at -80°C until analysis. The trial was registered at Clinicaltrials.gov (NCT02017444) and European Clinical Trials Database (EudraCT Number: 2013-003643-31) (K. A. Markey et al., 2017). The clinical parameters used for the calculation of correlations with the measured bile acids and ratios thereof are summarized in Supplemental Table S2 (details of their ascertainment have been previously published) (Hardy et al., 2021; K. A. Markey et al., 2017).
2.3. Sample Preparation

For the analysis of bile acids, 25 μL of serum (cohort A) or plasma (cohort B) were diluted 1:4 (v/v) with Milli-Q water. Samples were subjected to protein precipitation by adding 1 mL of 2-propanol containing a mixture of deuterated internal standards. The extraction was performed by continuous shaking for 30 min at 4°C at 1400 rpm on a Thermomixer C (Eppendorf AG, Hamburg, Germany) and then centrifuged at 16,000 × g for 10 min. Supernatants were transferred to new tubes and evaporated to dryness by using a Genevac EZ-2 system (SP Scientific, Warminster, PA, USA) at 35°C. The extracts were reconstituted with 100 μL of methanol to water (1:1, v/v), incubated at 4°C for 10 min at 1400 rpm, sonicated in a water bath for 10 min at room temperature, and finally transferred to LC-MS vials for analysis (Gómez et al., 2020).

2.4. LC-MS/MS analysis

Samples were analyzed by LC-MS/MS, consisting of an Agilent 1290 UPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent Technologies, Basel, Switzerland). Drying gas as well as nebulizing gas was nitrogen. The desolvation gas flow was set at 15 L/min, the sheath gas flow at 11 L/min, and the nebulizer pressure at 20 psi. The nitrogen desolvation temperature was set at 290°C, sheath gas temperature at 250°C. Capillary voltage was optimized for each segment from 2000 to 5000 V. Nozzle voltage was set at 2000 V and cell accelerator voltage at 5 V. Chromatographic separation of the bile acids was achieved using reversed-phase column (ACQUITY UPLC BEH C18, 1.7 mm, 2.1 μm, 150 mm, Waters, Wexford, Ireland). The flow rate was set at 0.5 mL/min and the column temperature at 55°C. The mobile phase consisted of ultrapure water and acetonitrile (95:5, v/v) with 0.1% formic acid (solvent A) and acetonitrile and ultrapure water (95:5, v/v) with 0.1% formic acid (solvent B) (Gómez et al., 2020).

2.4.1. Bile acid profile

The following gradient pattern was used for the separation of bile acids: 0 min, 25% B; 3.1 min, 35% B; 9 min, 38% B; 15 min, 65% B; 18 min, 65% B; 20 min, 100% B; 22 min, 25% B; and additional 2 min post-run at initial conditions. The injection volume was set at 3 μL. Data acquisition was performed using multiple reaction monitoring (MRM) mode. At least two transitions (quantifier and qualifier transitions) were selected for each compound in positive or negative ESI mode depending on the compound. Collision energy was optimized for each transition as described earlier (Gómez et al., 2020).

2.4.2. Bile acid ratio-specific method

The separation of GUDCA, GCDCA, and G7oxoLCA was achieved with the following gradient pattern: 0 min, 35% B; 4 min, 55% B; 5.50 min, 100% B; for 1 min, 100% B; and additional 1.5 min
post-run at initial conditions. The injection volume was set at 5 µL. Data acquisition was performed using MRM mode.

2.4.3. Quantification of AZD4017

For the analysis of the 11β-HSD1 inhibitor AZD4017, the gradient applied was as follows: 0 min, 45% B; 5 min, 90% B; 5.5 min, 100% B; 9 min, 100% B; 9.5 min, 45% B. The injection volume was set at 2 µL. Data acquisition was performed in ESI positive mode with the following MRM transitions: m/z 420.2 to 321.1 (CE 29V) and m/z 420.2 to 279 (CE 37V).

2.5. Data Analysis and Statistics

For LC-MS/MS data, MassHunter Acquisition Software and Quantitative Analysis vB.07.01 (Agilent Technologies, Inc., Santa Clara, CA, USA) were used for quantification. The Kruskal-Wallis test and Dunn’s multiple comparison were used to analyze significance of differences between groups. Spearman rank correlation was used to evaluate correlation between different variables. Grubbs’ test was performed to determine outliers. Statistical significance was established at p < 0.05. Statistical analysis and graphs were performed using GraphPad Prism v5.02 (GraphPad Software, Inc., San Diego, CA, USA) and RStudio v1.4.1106 (RStudio, PBC, Boston, MA, USA). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology.

3. Results

3.1. Impact of pharmacological 11β-HSD1 inhibition on serum bile acid profiles assessed in healthy males (cohort A)

Loss of 11β-HSD1 activity in transgenic mice leads to increased serum bile acid levels (Weingartner et al., 2021). We therefore first aimed to assess the effects of 11β-HSD1 inhibition by AZD4017 on serum bile acid profiles in healthy male volunteers. This cohort A consisted of 29 healthy male volunteers, distributed randomly into placebo and AZD4017 treatment groups (Othonos et al., 2023). All participants were simultaneously treated with prednisolone. To cover a broad range of bile acids, a recently developed LC-MS/MS-based method (Gómez et al., 2020) was applied to quantify a series of unconjugated and conjugated bile acids in serum samples at baseline and Post-Administration for both placebo and AZD4017 treatment groups. The mean serum concentrations of major individual bile acids, the sums of unconjugated and conjugated, as well as the sum of all measured bile acids are shown in Table 1. No effects due to prednisolone treatment were observed. Furthermore, no significant changes were detected in the levels of the major bile acids analyzed following AZD4017 treatment. A trend decrease was observed for CA, CDCA and UDCA upon 11β-HSD1 inhibition. G7oxoLCA was below the limit of quantification (LOQ) of the method (Gómez et al., 2020) in most of the samples.
analyzed from the baseline and placebo groups. However, upon 11β-HSD1 inhibition, G7oxoLCA levels increased at least ten-fold. To estimate the GCDCA/G7oxoLCA and GUDCA/G7oxoLCA ratios, a value of LOQ/2 was assigned to samples that could not be quantified. The calculated ratios were significantly lower in the AZD4017 Post-Administration group compared to the other three groups (Table 1), reporting the 11β-HSD1 inhibition.

**Table 1:** Concentrations of individual bile acids in human serum from cohort A. Bile acids were quantified by LC-MS/MS. The GUDCA/G7oxoLCA and GCDCA/G7oxoLCA ratios were assessed as potential biomarkers of 11β-HSD1 inhibition. Values are expressed as mean ± SD (nM). * p < 0.05 for Placebo Baseline vs AZD4017 Post-Administration groups; † p < 0.05 for AZD4017 Baseline vs AZD4017 Post-Administration groups, and ‡ p < 0.05 for Placebo Post-Administration vs AZD4017 Post-Administration groups. # not all of the values were within the LOQ; the value LOQ/2 was assigned for samples that were below LOQ.

<table>
<thead>
<tr>
<th>Cohort A</th>
<th>Placebo Baseline (n=14) nM (mean ± SD)</th>
<th>AZD0417 Baseline (n=15) nM (mean ± SD)</th>
<th>Placebo Post-Administration (n=14) nM (mean ± SD)</th>
<th>AZD4017 Post-Administration (n=15) nM (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unconjugated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>458 ± 622.8</td>
<td>465 ± 1230</td>
<td>393 ± 826</td>
<td>181.1 ± 215.7</td>
</tr>
<tr>
<td>CDCA</td>
<td>419.5 ± 467</td>
<td>529.3 ± 1301</td>
<td>450.1 ± 770.2</td>
<td>163.3 ± 127.1</td>
</tr>
<tr>
<td>DCA</td>
<td>459.1 ± 440.2</td>
<td>761.3 ± 1641</td>
<td>481.6 ± 682.7</td>
<td>443.7 ± 332</td>
</tr>
<tr>
<td>UDCA</td>
<td>129.1 ± 219.8#</td>
<td>64.4 ± 100.7#</td>
<td>160.8 ± 391.3#</td>
<td>30.2 ± 30</td>
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<tr>
<td>LCA</td>
<td>13.4 ± 7.8</td>
<td>13.2 ± 7.9</td>
<td>12.6 ± 5</td>
<td>15.6 ± 8.7</td>
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<tr>
<td>7oxoLCA</td>
<td>11.4 ± 11.2#</td>
<td>6.8 ± 7.1#</td>
<td>9.9 ± 12.5#</td>
<td>5.2 ± 3.8#</td>
</tr>
<tr>
<td>12oxoLCA</td>
<td>9.7 ± 7#</td>
<td>24.3 ± 50.9#</td>
<td>13.2 ± 22.3#</td>
<td>21.9 ± 41.8#</td>
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<td><strong>Conjugated</strong></td>
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<tr>
<td>GCA</td>
<td>218.4 ± 233.9</td>
<td>127.4 ± 90.25</td>
<td>274.2 ± 239</td>
<td>261.3 ± 226.2</td>
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<td>GCDCA</td>
<td>853.8 ± 866.3</td>
<td>368.8 ± 230.2</td>
<td>943.7 ± 957</td>
<td>807.8 ± 620.2</td>
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<tr>
<td>GDCA</td>
<td>362.9 ± 560.3</td>
<td>214.3 ± 207.4</td>
<td>322.8 ± 302.2</td>
<td>578.2 ± 570.6</td>
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<tr>
<td>GUDCA</td>
<td>227.9 ± 474.9</td>
<td>52.2 ± 62.7</td>
<td>163.5 ± 258.3</td>
<td>88.8 ± 81.7</td>
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<tr>
<td>G7oxoLCA</td>
<td>0.4 ± 0.6#</td>
<td>0.2 ± 0#</td>
<td>0.5 ± 0.6#</td>
<td>4.1 ± 3.7**†‡</td>
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<td>GLCA</td>
<td>14.7 ± 12.7#</td>
<td>11.9 ± 12.7</td>
<td>13.2 ± 13.7#</td>
<td>23.9 ± 24.2</td>
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<td>TCA</td>
<td>20.7 ± 26.1#</td>
<td>11.3 ± 14.7#</td>
<td>18.3 ± 28.5#</td>
<td>26.3 ± 27.1†</td>
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<td>TCDCA</td>
<td>81.7 ± 97.4</td>
<td>40.7 ± 25.3</td>
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<td>TDCA</td>
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<td>19.2 ± 16.4</td>
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<td>TLCA</td>
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<td>1.2 ± 1#</td>
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<td>2.6 ± 2.3#</td>
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<tr>
<td><strong>Total Unconjugated</strong></td>
<td>1500 ± 1502</td>
<td>1864 ± 4238</td>
<td>1521 ± 2424</td>
<td>861 ± 606</td>
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<tr>
<td><strong>Total Conjugated</strong></td>
<td>1810 ± 2167</td>
<td>847 ± 542</td>
<td>1834 ± 1587</td>
<td>1919 ± 1548</td>
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<tr>
<td><strong>Total Bile Acids</strong></td>
<td>3311 ± 3369</td>
<td>2711 ± 4374</td>
<td>3355 ± 3396</td>
<td>2780 ± 1900</td>
</tr>
</tbody>
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**Ratios**

<table>
<thead>
<tr>
<th></th>
<th>Placebo Baseline (n=14)</th>
<th>AZD0417 Baseline (n=15)</th>
<th>Placebo Post-Administration (n=14)</th>
<th>AZD4017 Post-Administration (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCDCA/G7oxoLCA</td>
<td>3276 ± 3019</td>
<td>2002 ± 1315</td>
<td>2735 ± 1526</td>
<td>241 ± 98**†‡</td>
</tr>
<tr>
<td>GUDCA/G7oxoLCA</td>
<td>645 ± 782</td>
<td>284 ± 353</td>
<td>400 ± 267</td>
<td>24 ± 13*†‡</td>
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</tbody>
</table>

To achieve a higher sensitivity for the quantification of G7oxoLCA, allowing calculation of the GCDCA/G7oxoLCA and GUDCA/G7oxoLCA ratios, a second LC-MS/MS method was developed (see 2.4.2.). This method showed improved analytical sensitivity by including only the three bile acids
needed for the ratios as well as increasing dwell times and injection volume, allowing for shorter time-frame for analysis. All previously extracted samples were reanalyzed using this short and focused method, and G7oxoLCA could be successfully quantified in all samples. The mean concentrations of the three bile acids and of the GCDCA/G7oxoLCA and GUDCA/G7oxoLCA ratios are shown in Supplemental Table S3. G7oxoLCA levels were significantly increased in AZD4017 Post-Administration group when compared to other three groups. Furthermore, the GCDCA/G7oxoLCA and GUDCA/G7oxoLCA ratios were both significantly decreased in the AZD4017 Post-Administration compared to the other groups (Figure 2A and 2B, Supplemental Table S3).

Several clinical parameters determined in serum and over-night urine samples were collected from the participants (Supplemental Table S1). Serum lipids (cholesterol, high-density lipoprotein (HDL), triglycerides) and markers of liver function (bilirubin, albumin, gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP)) were not different between the four groups and ADZ4017 treatment did not affect these parameters. Also, the serum levels of the gonadal steroid testosterone and of the sex hormone binding globulin (SHBG) did not differ among the four groups. In contrast, the serum concentrations of the adrenal androgens androstenedione and DHEAS and of the glucocorticoid cortisol as well as that of the urinary glucocorticoid metabolites decreased significantly in the placebo Post-Administration group compared to the two baseline groups. It needs to be noted that both placebo and AZD4017 treatment groups received prednisolone, which suppresses adrenal steroidogenesis. This is supported by the lower serum levels of ACTH in the placebo Post-Administration group. AZD4017 treatment reversed the decreased serum ACTH and adrenal androgen levels as well as those of cortisol and cortisone in 24-hour urine samples. Importantly, whilst the 11β-HSD2 activity marker, i.e., urinary cortisol/cortisone ratio, was not altered by AZD4017 treatment, both urinary biomarkers of 11β-HSD1 activity, i.e. (αTHF +THF)/THE and THF/THE were decreased by an order of magnitude, indicating 11β-HSD1 inhibition (Fig. 2C and D, Suppl. Table S1).

**Figure 2:** Serum bile acid ratios and urine glucocorticoid metabolite ratios as biomarkers of 11β-HSD1 inhibition. The ratios GCDCA/G7oxoLCA (A) and GUDCA/G7oxoLCA (B) in human serum from cohort A were determined by quantification of the respective bile acids by LC-MS/MS. The ratios THF/THE (C) and (αTHF+THF)/THE (D) in over-night urine samples from cohort A were determined by GC-MS as reported earlier (Othonos et al., 2023). Values are expressed as mean ± SD. *** p ≤ 0.001.
The parameters reported previously ((Othonos et al., 2023), Suppl. Table S1) were used to test possible correlations with AZD4017 treatment and to assess the efficacy of the proposed bile acid ratio as biomarker of 11β-HSD1 inhibition. Possible correlations between the GUDCA/G7oxoLCA and GCDC/G7oxoLCA ratios with the clinical variables shown in Supplemental Table S1 were investigated (see Suppl. Table S4). As expected, no correlations and no influence of any parameter on these two bile acid ratios could be found for the placebo and AZD4017 baseline groups. Importantly, Spearman correlations between the GUDCA/G7oxoLCA ratio and the urinary THF/THE and αTHF+THF)/THE ratios revealed strong positive associations, with $r = 0.81$ and $r = 0.85$, respectively, when comparing placebo and AZD4017 Post-Administration groups. Furthermore, GUDCA/G7oxoLCA correlated negatively with serum DHEAS ($r = -0.58$) and androstenedione ($r = -0.64$) and with urinary cortisone ($r = -0.65$) and cortisol ($r = -0.69$). Moreover, the concentration of the inhibitor AZD4017 was used to evaluate its influence on the observed bile acid changes and as
predicted, a negative correlation was obtained ($r = -0.77$). Similar results were obtained for the GCDCA/G7oxoLCA ratio (Suppl. Table S4).

3.2. Validation of the bile acid ratios as biomarkers of $11\beta$-HSD1 inhibition in females with IIH (cohort B)

To confirm the predictive value of the GUDCA/G7oxoLCA and GCDCA/G7oxoLCA ratios for the pharmacological inhibition of $11\beta$-HSD1, a second clinical cohort B, consisting of a group of 29 women diagnosed with active IIH (ICP >25 cmH₂O) and active papilledema, was investigated. Plasma samples from cohort B, collected before and after administration of placebo or AZD4017, were analyzed by LC-MS/MS to determine bile acid profiles (Gómez et al., 2020). The mean concentrations of individual bile acids and the sums of unconjugated, conjugated, and all measured bile acids are shown in Table 2. With the exception of G7oxoLCA, which was significantly increased in the AZD4017 Post-Administration group, there were no significant changes in any of the bile acids analyzed when comparing the four groups. G7oxoLCA levels were below LOQ in most of the samples analyzed, except in the AZD4017 Post-Administration group; and to estimate the GCDCA/G7oxoLCA and GUDCA/G7oxoLCA ratios shown in Table 2, the value LOQ/2 was used for the samples that could not be quantified. Both calculated ratios were significantly decreased in the AZD4017 Post-Administration group compared to the other groups (Table 2).

Table 2: Concentrations of individual bile acids in plasma of clinical cohort B. Bile acids were quantified by LC-MS/MS (Gómez et al., 2020). The GUDCA/G7oxoLCA and GCDCA/G7oxoLCA ratios were tested as potential biomarkers of $11\beta$-HSD1 inhibition. Values are expressed as mean ± SD (nM). * p < 0.05 Placebo Baseline vs AZD4017 Post-Administration groups; † p < 0.05 AZD4017 Baseline vs AZD4017 Post-Administration groups, and ‡ p < 0.05 Placebo Post-Administration vs AZD4017 Post-Administration groups. # not all of the values were within the LOQ; the value LOQ/2 was assigned for samples that were below LOQ.

<table>
<thead>
<tr>
<th>Cohort B</th>
<th>Placebo Baseline (n=12) nM (mean ± SD)</th>
<th>AZD4017 Baseline (n=17) nM (mean ± SD)</th>
<th>Placebo Post-Administration (n=12) nM (mean ± SD)</th>
<th>AZD4017 Post-Administration (n=17) nM (mean ± SD)</th>
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<tr>
<td>Unconjugated</td>
<td>CA</td>
<td>149 ± 274.6</td>
<td>92.61 ± 73.95</td>
<td>114.1 ± 151.9</td>
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<tr>
<td></td>
<td>CDCA</td>
<td>260 ± 363</td>
<td>157.3 ± 133.5</td>
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<td></td>
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<td>454.2 ± 405.5</td>
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<td>79.1 ± 91.1#</td>
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<td>3.2 ± 0#</td>
<td>6.7 ± 5.4#</td>
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<td>12oxoLCA</td>
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<td>Conjugated</td>
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<td>190.8 ± 129.3</td>
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<td>0.2 ± 0#</td>
<td>0.2 ± 0#</td>
<td>4.4 ± 6.7*†‡</td>
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<td>GLCA</td>
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<td>Total Conjugated</td>
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<td>Total Bile Acids</td>
<td>3791 ± 2595</td>
<td>2526 ± 1760</td>
<td>2619 ± 1194</td>
<td>3019 ± 1810</td>
</tr>
</tbody>
</table>

**Ratios**

| GCDCA/G7oxoLCA | 7040 ± 6746 | 4811 ± 4889 | 4429 ± 3676 | 1653 ± 2410*†‡ |
| GUDCA/G7oxoLCA | 1318 ± 1784 | 629 ± 535 | 1067 ± 723 | 208 ± 501*†‡ |

To reach sufficient sensitivity for quantification of G7oxoLCA also in the control groups, the shorter and focused LC-MS/MS method was applied. The mean concentrations of GUDCA, GCDCA and G7oxoLCA and the GUDCA/G7oxoLCA and GCDCA/G7oxoLCA ratios are shown in Supplemental Table S5. G7oxoLCA levels were significantly increased in the AZD4017 Post-Administration group compared to the three other groups. Additionally, the GCDCA/G7oxoLCA and GUDCA/G7oxoLCA ratios were significantly decreased in the AZD4017 Post-Administration group compared to the other groups (Figure 3A and 3B, Supplemental Table S5).

Different clinical parameters measured in serum of cohort B were reported earlier (Hardy et al., 2021; K. Markey et al., 2020) and are shown in Supplemental Table S2. Blood lipids and parameters of liver function were not different among the four groups. However, whilst concentrations of serum androgens and glucocorticoids were comparable between the four groups, there was a trend decrease of the cortisol/DHEA and cortisol/cortisone ratios in the ADZ4017 treatment group, along with significantly decreased urinary THF/THE and (αTHF+THF)/THE ratios as markers of 11β-HSD1 activity (Figure 3C and 3D, Supplemental Table S2) (K. Markey et al., 2020; K. A. Markey et al., 2017). The cortisone metabolite THE was significantly increased and THF and αTHF tended to be decreased in 24-hour urine samples of the AZD4017 Post-Administration group compared to the baseline and placebo treated groups.

**Figure 3:** Plasma bile acid ratios and urine glucocorticoid metabolite ratios as biomarkers of 11β-HSD1 inhibition. The ratios GCDCA/G7oxoLCA (A) and GUDCA/G7oxoLCA (B) in human plasma from cohort B were determined by quantification of the respective bile acids by LC-MS/MS. The ratios THF/THE (C) and (αTHF+THF)/THE (D) in 24-hour urine samples from cohort B were determined by GC-MS as reported earlier (K. Markey et al., 2020). Values are expressed as mean ± SD. Two non-responders are highlighted in red. * p ≤ 0.05, ** p ≤ 0.01, and *** p ≤ 0.001.
Possible correlations of the GCDCA/G7oxoLCA and GUDCA/G7oxoLCA ratios with the available clinical parameters shown in Supplemental Table S2 (K. Markey et al., 2020) were examined (see Suppl. Table 6). As expected, no correlations could be found between the two bile acid ratios and any of the clinical parameters for the placebo and AZD4017 baseline groups. Then, Spearman correlations between the two bile acid ratios and the remaining variables were investigated in both Post-Administration groups (placebo vs AZD4017). The GUDCA/G7oxoLCA ratio correlated positively with the urinary ratios THF/THE ($r = 0.77$) and ($\alpha$THF+THF)/THE ($r = 0.73$), and the serum ALP ($r = 0.50$) and cortisol/cortisone ratio ($r = 0.58$). Moreover, the concentration of the inhibitor AZD4017 correlated negatively with the GUDCA/G7oxoLCA ratio ($r = -0.76$). Similar results were obtained for the GCDCA/G7oxoLCA ratio (Suppl. Table S6).

### 3.3. Quantification of AZD4017 concentrations in cohorts A and B

To detect potential outliers in the treatment group, an LC-MS/MS method was established for the quantification of the 11β-HSD1 inhibitor AZD4017 in the placebo and AZD4017 Post-Administration groups of cohort A and B. As expected, the inhibitor was absent in all samples from the placebo Post-
Administration groups. In the AZD4017 Post-Administration group of cohort A the 11β-HSD1 inhibitor was detected in all serum samples (n = 15), with concentrations of 894 ± 497 nM (mean ± SD). In cohort B, the inhibitor could be detected in all but two plasma samples from individuals of the AZD4017 Post-Administration group (n = 17), with concentrations of 1384 ± 1152 nM (mean ± SD). The two participants of the AZD4017 Post-Administration group with non-detectable blood inhibitor levels also showed the highest bile acid and tetrahydro-glucocorticoid metabolite ratios of their group, as highlighted in red in Figure 3 and in Supplemental Figure S1, and marked as ND in Figure 4.

3.4. Robustness of the proposed bile acid ratio biomarkers of 11β-HSD1 activity

To analyze the robustness of the proposed bile acid ratio biomarkers of 11β-HSD1 activity, the results obtained from cohort A and cohort B were combined. Despite the considerable differences in the design of the two cohorts in terms of sex, health state, BMI, time of treatment, co-treatment, and matrix to be analyzed, the blood bile acid ratios GCDCA/G7oxoLCA and GUDCA/G7oxoLCA as well as the THF/THE and (αTHF+THF)/THE ratios determined in 24-hour urine samples were significantly decreased in the AZD4017 Post-Administration groups of cohorts A and B and of the combined data thereof, even without excluding the two outliers of cohort B marked in red (Fig. 3, Supplemental Figure S1).

Analysis of the data from the placebo and AZD4017 Post-Administration groups from both cohorts A and B revealed a strong correlation (r = 0.77) (Figure 4A). The values from the AZD4017 Post-Administration group clustered together, with the exception of the two individuals with non-detectable plasma AZD4017 levels (marked as ND). Additionally, a principal component analysis (PCA) was performed by combining the data of all samples from cohorts A and B and from baseline and Post-Administration groups. All available clinical parameters, the urinary tetrahydro-glucocorticoid ratios and the bile acid ratios were used for the analysis (Figure 4B). The PCA analysis clearly separated the ADZ4017 Post-Administration group from the other three control groups. The two outliers with non-detectable drug levels are indicated as ND and they clustered with the control groups.
Figure 4: Contrasting data from AZD4017 treated individuals to those of untreated controls. (A) Spearman correlation of the urinary (αTHF+THF)/THE ratio and the blood GUDCA/G7oxoLCA ratio ($r = 0.77$), combining data from placebo and AZD4017 Post-Administration groups from both cohorts A and B. Two individuals with non-detectable AZD4017 levels are marked by ND. (B) Principal component analysis (PCA) built by combining all samples from both cohorts A and B, Baseline and Post-Administration groups from both placebo and AZD4017-treated patients. All variables available from both cohorts A and B and the urinary tetrahydro-glucocorticoid ratios and bile acid ratios were used for the analysis. Two individuals with non-detectable AZD4017 levels are marked by ND.
4. Discussion

Inhibition of 11β-HSD1 is considered for treatment of various diseases including metabolic disorders, atherosclerosis, idiopathic intracranial hypertension (IIH), skin diseases, osteoporosis, cognitive impairments in aging, and optimization of glucocorticoid therapy to ameliorate adverse effects in metabolic target organs (Anderson & Walker, 2013; Bianzano et al., 2021; Chuanxin et al., 2020; Courtney et al., 2008; Feig et al., 2011; Freude et al., 2016; Gregory et al., 2020; Heise et al., 2014; K. Markey et al., 2020; Othonos et al., 2023; Schwab et al., 2017; Shah et al., 2011; Stefan et al., 2014; Webster et al., 2017). Non-invasive biomarkers to assess the activity of a given target can greatly facilitate the evaluation of drug efficacy. With respect to 11β-HSD1, ratios of urinary glucocorticoid metabolites, i.e. THF/THE and (5αTHF + THF)/THE, are usually used in clinical studies to assess the efficacy of inhibitors of this enzyme (Bianzano et al., 2021; Courtney et al., 2008; Freude et al., 2016; Jamieson et al., 1999; K. Markey et al., 2020; Sagmeister et al., 2019; Tomlinson & Stewart, 2001; Webster et al., 2017). However, this requires an additional intervention, namely the collection of 24-hour urine samples, and leads to a higher burden for the patient and additional costs. A blood-based biomarker is currently not available; however, a recent preclinical study using four different mouse models proposed the use of the bile acid ratio TUDCA/T7oxoLCA as a blood biomarker for genetically or pharmacologically decreased 11β-HSD1 oxoreductase activity (Weingartner et al., 2021). As a next step, the present study aimed at translating this bile acid ratio biomarker from mouse to human and it also assessed whether 11β-HSD1 inhibition would result in pronounced disturbances of the plasma bile acid profile.

Two clinical studies of pharmacological 11β-HSD1 inhibition were analyzed. Both used the same inhibitor but differed in several important parameters, i.e. health state (IIH vs healthy), body weight (obese vs normal weight), sex, co-medication (w/wo prednisolone), and matrix of the sample (serum vs plasma). Despite the differences in these parameters, the bile acid profiles in both cohorts revealed no gross alterations following 11β-HSD1 inhibition, with the exception of significantly increased levels of the secondary bile acid G7oxoLCA. Earlier studies using transgenic mice lacking 11β-HSD1 activity observed elevated plasma bile acid concentrations (Penno et al., 2014; Weingartner et al., 2021), raising potential concerns for a higher risk of cholestasis upon inhibition of 11β-HSD1. Additionally, changes in the composition of the gut microbiota have been observed in 11β-HSD1 KO mice that could account for alterations in bile acid homeostasis (Johnson et al., 2017). The present study, including two different clinical cohorts and focusing on pharmacological inhibition, does not support such concerns, and the increase in G7oxoLCA was the major change in the bile acid profile following 11β-HSD1 inhibition. In humans, glycine conjugation is preferred over taurine conjugation, and T7oxoLCA was below the quantification limit. Whilst the bile acid profiling method allowed for quantification of G7oxoLCA in samples from AZD4017 treated individuals, it was not sensitive enough to analyze this bile acid in the control groups. Thus, a shorter and focused LC-MS method specifically covering G7oxoLCA, GUDCA
and GCDCA was developed and applied, enabling quantification of G7oxoLCA also in most of the samples from the control groups. For the few samples that still were undetectable or showed values below LOQ, the value LOQ/2 was included to calculate the ratios.

Although the inhibition of 11β-HSD1 resulted in increased G7oxoLCA blood levels, there are typically large interindividual variations when looking at a specific bile acid metabolite, likely due to differences in food intake and composition. Other factors such as age, sex, health state or co-medication can also influence the concentration of a specific bile acid. For these reasons product/substrate ratios are considered more reliable and robust biomarkers reporting altered enzyme activity. This was demonstrated by a recent preclinical study in 11β-HSD1 KO mice (Weingartner et al., 2021), where TUDCA/T7oxoLCA was shown to robustly detect genetically and pharmacologically diminished 11β-HSD1 activity, whereas the concentration of T7oxoLCA alone was elevated but showed high interindividual variation and was therefore not an ideal biomarker for the impaired enzyme activity. Thus, the bile acid ratios GUDCA/G7oxoLCA and GCDCA/G7oxoLCA were analyzed as biomarkers of 11β-HSD1 oxoreductase activity in this study. While both ratios robustly detected the 11β-HSD1 inhibition, the GUDCA/G7oxoLCA ratio seemed to perform better. This may be explained by the fact that 7oxoLCA and its conjugated forms are preferentially converted to UDCA rather than CDCA and the respective conjugated metabolites (Odermatt et al., 2011; Penno et al., 2013). Furthermore, both 7oxoLCA and UDCA are secondary bile acids, whereas CDCA is a primary bile acid that is regulated by distinct physiological pathways.

A comparison of the use of the blood GUDCA/G7oxoLCA ratio with the 24-hour urine THF/THE and (5αTHF+THF)/THE ratios showed that they all robustly detected the pharmacologically inhibited 11β-HSD1 activity. For both cohorts A and B, a positive correlation (r > 0.7) was found between the blood bile acid ratios and the urinary tetrahydro-glucocorticoid ratios tested. Importantly, quantification of blood AZD4017 levels revealed two individuals in cohort B with undetectable drug levels, suggesting either insufficient patient compliance or very rapid drug metabolism, and both showed blood bile acid ratios and 24-hour urine tetrahydro-glucocorticoid ratios within the normal range. Interestingly, in cohort A, but not in cohort B, the GUDCA/G7oxoLCA ratio correlated negatively with serum DHEAS, serum androstenedione and urinary cortisone and cortisol concentrations (r < -0.6). In cohort A, AZD4017 was administered in combination with prednisolone, whereas the control group received only prednisolone. Prednisolone can be converted to the inactive prednisone by 11β-HSD2, mainly in the kidney, and regenerated by 11β-HSD1 in the liver and adipose. Prednisolone suppresses adrenal steroidogenesis via negative feedback regulation. Inhibition of 11β-HSD1 partially reversed the glucocorticoid-mediated suppression of adrenal steroidogenesis, explaining the negative correlation between these adrenal steroids and the blood bile acid and urinary tetrahydro-glucocorticoid ratios. To further assess the robustness of the GUDCA/G7oxoLCA ratio as a biomarker of pharmacological 11β-HSD1 inhibition, the data from both clinical cohorts were combined. Regardless of the differences
between the two cohorts in terms of sex, health condition, BMI, co-medication or sample matrix, the values of the blood bile acid and urinary tetrahydro-glucocorticoid ratios obtained from both cohorts were in the same range and successfully detected the decreased 11β-HSD1 activity.

In conclusion, the present study proposes the GUDCA/G7oxoLCA ratio as a biomarker to detect pharmacological inhibition of 11β-HSD1. The main advantages offered by this blood bile acid ratio biomarker include its non-invasiveness, the need for small sample volume (25 µL of either plasma or serum that can be stored at -80°C until use), specificity and sensitivity due to mass spectrometry-based quantification, and that it is not influenced by 11β-HSD2, which does not accept CDCA, UDCA and their conjugates as substrates. There are several potential limitations that need to be addressed in future studies: 1) the GUDCA/G7oxoLCA ratio may mainly represent hepatic 11β-HSD1 oxoreductase activity. Whether decreased 11β-HSD1 oxoreductase activity specifically in skeletal muscle or adipose can also be detected remains to be determined, 2) it needs to be assessed whether co-medication, different diet or disease states can differentially affect GUDCA and G7oxoLCA, thereby lowering the informative value of the ratio, and 3) the GUDCA/G7oxoLCA ratio should be tested in disease situations where 11β-HSD1 and/or H6PD are downregulated to see whether it can detect the decreased oxoreductase activity.

**Supplementary Materials:** The following are available online at xxx, Table S1: Clinical parameters of participants from cohort A. Table S2: Clinical parameters of participants from cohort B. Table S3: Concentrations of GUDCA, GCDC and G7oxoLCA in serum from clinical cohort A. Table S4: Spearman’s correlation coefficients (r values) between the GCDC/G7oxoLCA and GUDCA/G7oxoLCA ratios and the clinical variables. Table S5: Concentrations of GUDCA, GCDC and G7oxoLCA in plasma samples from clinical cohort B. Table S6: Spearman’s correlation coefficients (r values) between the GCDC/G7oxoLCA and GUDCA/G7oxoLCA ratios and the clinical variables. Figure S1: Combined data of bile acid and tetrahydro-glucocorticoid metabolite ratios of cohort A and B.

**Author Contributions:** Conceptualization, C.G. and A.O., Methodology, C.G., Z.A. and N.O.; Data Acquisition and Analysis, C.G., Z.A. and N.O.; Validation, C.G.; Writing – Original Draft Preparation, C.G.; Writing – Review & Editing, C.G., Z.A., N.O., D.V.W., S.W. G.L., J.W.T., A.J.S. and A.O.; Supervision, A.O.; Project Administration, A.O.; Funding Acquisition, A.O.

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**Conflicts of Interest Statement:** A.J.S. reports personal fees from Invex therapeutics in her role as Director with stock holdings as well as personal fees from Allergan, Novartis, Cheisi and Amgen (unrelated to the submitted work).

**Data Availability Statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request. All related study data will be provided according to the related data management plan as open access at https://zenodo.org/.

5. **References**


