

Supplementary Text 2: Steroid quantification of hormones in plasma or serum samples – Stero17+2

The analysis of steroids in plasma or serum have been performed in the Metabolomics Platform of the Genome Analysis Center, Helmholtz Zentrum München. The following 19 steroids were quantified using an extended version of the Absolute*IDQ*TM Stero17 Kit and LC-ESI-MS/MS: aldosterone, androstenedione (androst-4-en-3,17-dione), androsterone, corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate (DHEAS), dihydrotestosterone (DHT), estradiol, estrone, etiocholanolone, 17 α -hydroxyprogesterone, progesterone, testosterone, pregnenolone, pregnanediol (last two steroids were assessed semiquantitative). Compound identification and quantification were based on scheduled multiple reaction monitoring measurements (sMRM). Sample preparation and LC-MS/MS measurements were performed as described by the manufacturer in manual UM-STERO17¹. The method of the Absolute*IDQ*TM Stero17 Kit has been proven to be in conformance with the EMEA-Guideline "Guideline on bioanalytical method validation (July 21st 2011)"², which implies proof of reproducibility within a given error range. Analytical specifications for LOD (limit of detection), LLOQ and ULOQ (lower and upper limit of quantification), specificity, linearity, precision, accuracy, reproducibility, and stability were determined experimentally by Biocrates and are described in the manual AS-STERO17. A detailed method description has been published³.

We provide the description on how the method was implemented in the laboratory:

Plasma or serum samples have been prepared in the group of the collaboration partner. All samples have been stored at -80 °C until sample preparation for measurements. In the metabolomics laboratory, 400 μ L of ultrapure water were pipetted into each well of a 2-mL-

96-well deep well plate. Except into the blank well, 20 μ L of the internal standard mix were added to each well. Thereafter, 250 μ L of blank, calibration standards, quality control samples, and plasma or serum samples were pipetted into the distinct respective wells. The well content was mixed by aspiration using robot-driven pipets. In between, the SPE (solid phase extraction) plate of the kit was conditioned successively with 1 mL of dichloromethane, followed by 1 mL acetonitrile, 1 mL methanol, and 1 mL ultrapure water. Except for sample loading, all SPE purification steps (conditioning, washing, drying, and eluting) were done by pressing solvents through the SPE plate using nitrogen and the positive pressure unit. The velocity was regulated by variation of the nitrogen pressure. After plate conditioning, the mixed samples were loaded onto the SPE plate. The samples dropped through very slowly by gravitation (1-2 drops per second). The SPE plate was washed with 500 μ L water, dried for 1 h under nitrogen stream (58 psi). Steroids were subsequently eluted in two steps: 1) Two times with 500 μ L dichloromethane into the same deep well plate (all steroids except DHEAS eluted), the eluate was dried each time for 20 min at 45 psi. 2) With 600 μ L acetonitrile into another deep well plate. The first dichloromethane fraction was dissolved in 50 μ L of methanol/water (25/75 v/v) and the plate was covered with a lid. To facilitate dissolving, the plate was treated for 1 min in an ultrasonic bath, and afterwards shaken for 5 min at 600 rpm. The second acetonitrile fraction was diluted with 400 μ L of water and after placing a lid the plate was treated like the dichloromethane fraction. Both plates were centrifuged at 50 x g and placed into the cooled auto sampler (10 °C) for LC-MS/MS measurements. The LC-separation of both fractions was performed using 470 mL ultrapure water and the content of three ampules of the kit as mobile phase A and acetonitrile/methanol/ultrapure water v/v/v 85/10/5 as mobile phase B. Steroids were separated on the HPLC column for Absolute*IDQ*TM Stero17 Kit combined with the precolumn SecurityGuard Cartridge C18 4 x 2 mm (for HPLC, Phenomenex Cat No. AJ0-

4286). All solvents that have been used for sample preparation and measurement were of HPLC grade. Samples were handled using a Hamilton Microlab STARTM robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a Waters Positive Pressure-96 Processor (Waters GmbH, Eschborn, Germany), beside standard laboratory equipment. Mass spectrometric analyses were done on a QTRAP 5500 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1260 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.2. Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the software MultiQuant 3.0.1 (Sciex) and the MetIDQTM software package, which is an integral part of the AbsoluteIDQTM Stero17 Kit. Metabolite concentrations were calculated using internal standards and reported in nM or ng/mL.

References

1. Biocrates (2019). AbsoluteIDQ Stero17 Kit. Increased Confidence in Steroid Hormone Analysis. Available at: <https://www.biocrates.com/products/research-products/absoluteidq-stero17-kit> [Accessed June 13, 2019].
2. Committee for Medicinal Products for Human Use (CHMP). Guideline on bioanalytical method validation. EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2, 21 July 2011.
3. Breier M, Wahl S, Prehn C, Ferrari U, Sacco V, Weise M, Gallert H, Adamski J, Lechner A (2017) Immediate reduction of serum citrulline but no change of steroid profile after initiation of metformin in individuals with type 2 diabetes. *J Steroid Biochem Mol Biol.*