**DATA SUPPLEMENT**

**„ Endothelial epoxyeicosatrienoic acid release is intact in aldosterone excess”**

**Supplemental Materials and Methods:**

**Patient cohort**

Case definition of primary aldosteronism:

Patients were recruited at the day of confirmatory testing for workup of arterial hypertension when results were still pending.

Patients underwent saline infusion test (SIT, 2 L NaCl 0.9 % administered i.v. in 4 hours) and/or 2 h captopril challenge test (CCT, 50 mg captopril administered orally) under non-interfering medication (usually doxazosin ± verapamil). PA criteria were the following:

1. plasma aldosterone baseline ≥50 pg/mL AND suppressed direct renin concentration (<12 µU/mL) AND aldosterone/renin ratio (ARR) ≥12 pg/mL:µU/mL

2. AND post SIT aldosterone ≥50 pg/mL AND DRC <12 µU/mL

3. OR post CCT aldosterone ≥70 % of baseline aldosterone AND DRC <12 µU/mL OR ARR ≥12 pg/mL:µU/mL

In case of equivocal results in both tests, the SIT result was regarded as definitive. Information about lateralization was obtained by adrenal venous sampling. When DRC was below 2 µU/mL, the lower limit of quantification (LLQ), the LLQ value was used to compute ARR. All patients with excluded primary aldosteronism were classified as essential hypertension (EH).

PROCAM risk score was calculated via an excel sheet using the original formula [1].

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. LMU Munich ethics committee approved the study protocol. Written informed consent was obtained from all participants included in the study. Data protection and privacy laws were adhered to.

**Cell culture and stimulation**

Human coronary artery endothelial were purchased from Lonza (Basel, CH) together with their specific growth media and growth factors and were cultured in specific growth-medium with supplements according to the manufacturer’s recommendations (details see below in section Drugs, buffer compositions, chemicals). All experiments were conducted between passages 5-9 and both cell lines were maintained at 37°C, 5% CO2 and 95% O2 in a humidified incubator. Cells were serum starved over night before treatment in EBM-2 medium as the confluence reached 85-90%. Pharmacological treatment was performed in serum-free medium for an additional 48 hours.

**RNA extraction, reverse transcription and qPCR**

Total RNA was extracted with a cartridge-based Maxwell 16 LEV simply RNA cells kit (Promega, Walldorf, Germany) following to manufacturer’s protocol. This protocol includes treatment of the lysates with DNAse I to remove possible contamination with genomic DNA. RNA concentration was measured with a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Munich, Germany). For cDNA synthesis, RNA was reversed transcribed via the GoScript™ Reverse Transcriptase Kit (Promega,). Quantitative real-time PCR (qRT-PCR) was conducted on the QuantStudio 5 systerm (Thermo Fisher Scientific) using specific Taqman probes (see Supplementary Table S1 below). Relative expression levels of each gene of interest were normalized to the geometric mean of two housekeeping genes (EIF2B1 and HPRT1).

|  |  |
| --- | --- |
| **Gene of interest** | **Taqman probe ID** |
| CYP2C8 | Hs00946140\_g1 |
| CYP2C9 | Hs04260376\_m1 |
| CYP2J2 | Hs00559374\_m1 |
| EPHX1 | Hs01116806\_m1 |
| EPHX2 | Hs00932316\_m1 |
| EIF2B1 | Hs00426752\_m1 |
| HPRT1 | Hs99999909\_m1 |
| HSD11B1 | Hs01547870\_m1 |
| HSD11B2 | Hs00388669\_m1 |
| SCNN1A | Hs00168906\_m1 |

**Supplementary Table S1**: Genes of interest and corresponding Taqman probes used in the qPCR experiments.

**RNAseq and bioinformatics**

RNAseq and downstream bioinformatics were performed by a commercial partner (Eurofins Genomics, Ebersberg, Germany). Raw FASTQ files were depleted of rRNA reads using RiboDetector [3] and underwent adapter trimming, quality filtering, per-read quality pruning and removal of poor quality bases using fastp software [2]. Sequences were then aligned and mapped to hg38 using STAR software [5] followed by transcript quantification by RSEM [6]. Differential gene expression was analysed using the DESeq2 package in R/Bioconductor with the Benjamini-Hochberg method to correct for multiple testing [7].

Read counts of *VCAM1* and *CHRNE* were normalized to the geometric mean of the read counts of *EIF2B1* and *HPRT1*.

**Fluo4-based calcium imaging**

ECs were treated as described above in a 96-well plate (Corning® 96 Well CellBIND® Microplates, Sigma Aldrich), loaded with Fluo-4 (5 µM) in HEPES buffer supplemented with 0.1 % pluronic (v/v) (Thermo Fisher Scientific) for 1 hour at 37 °C. Fluo-4 fluorescence signals were acquired with a victor X4 plate reader (PerkinElmer, Walluf, Germany) equipped with injectors to register time-resolved fluorescence after injection. Cells which had undergone the same treatment regimens but which had not been loaded with Fluo4 were included in each run and stimulated with the same acetylcholine concentrations to determine non-specific background fluorescence for each acetylcholine concentration and treatment. These values were subtracted from the raw fluorescence readings. Background-corrected fluorescence (F) at each timepoint was then normalized to the initial background-corrected fluorescence (F0) to yield F/F0.

**Drugs, chemicals, cells, buffer compositions**

Drugs and chemicals

Acetylcholine: A2661, Sigma Aldrich, Taufkirchen, Germany

Aldosterone: A9477, Sigma Aldrich, Taufkirchen, Germany

Eplerenone: 2397, Tocris, Bristol, UK

Fluo-4: 6255, Tocris, Bristol, UK

GSK2256294: 2220, Axon Medchem BV, Groningen, The Netherlands

Hydrocortisone: H0888, Sigma Aldrich, Taufkirchen, Germany

Cells, culture media, growth factors

Endothelial cells: Clonetics™ coronary artery endothelial cells, Lonza, Basel, CH

Endothelial medium: 1xEBM™ 2 Basal Medium（CC-3156, Lonza)

Endothelial growth factors: 1xEGM™-2 MV SingleQuots™ Supplement Pack (CC-4147, Lonza) containing: FBS 25ml, Hydrocortisone 0.2ml, hFGF-B 2ml, VEGF 0.5ml, R3-IGF-1 0.5ml, Ascorbic Acid 0.5ml, hEGF 0.5ml, GA 1000 (=gentamicin + amphotericin B) 0.5ml.

Buffers

HEPES buffer (in mM): NaCl 140, KCl 5,4, MgCl2 1, HEPES 10, Glucose 10, CaCl2 x 2H2O 2; pH ad 7,4 with NaOH

**Targeted LC-MS/MS analysis of oxylipins**

Compounds

All compounds were purchased from Cayman Chemicals.

Sample preparation for LC-MS/MS

All steps of the extraction procedure were conducted at pre-cooled temperature with tubes or plates in wet ice, except solid phase extraction (SPE).

Preparation of tissue samples and quality controls:

100 µL of cell culture supernatant or 50 µL of human plasma samples were transferred into a 1.5 mL Eppendorf tube. QC pool samples were prepared in triplicates by taking out 50 µL from each study sample. The pool sample was subsequently mixed and 50 µL (plasma) or 100 µL (cell supernatant) were transferred into 1.5 mL Eppendorf tubes.

QC reference samples were prepared in triplicates in 1.5 mL Eppendorf tubes by mixing 5 µL of the standard mixture (30 ng/mL for oxylipins and 300 ng/mL for PUFAs) with 45 µL of water. Blank (triplicate) and zero (single) samples were prepared by transferring 50 µL of H2O (milliQ) into 1.5 mL Eppendorf tubes. Calibrators were prepared in 1.5 mL Eppendorf tubes by successive dilutions (factor 3) in water/MeOH (50:50, v/v) of the calibration mixture (200 ng/mL for oxylipins and 2000 ng/mL for PUFAs) to reach 9 calibrator points (cal.): 66.67 ng/mL (cal. 09) to 0.010 ng/mL (cal. 01). 30 µL of each cal. was then transferred to a new 1.5 mL Eppendorf tube.

For accurate quantification, 10 µL of 5 ng/mL (50 ng/mL for PUFAs) ISTD mixture were added to the samples, except zero sample.

Extraction procedure:

For lipid extraction, 150 µL of cold MeOH (-20 °C) were added to the samples followed by incubation for 10 min with vortexing every 3 min. Protein precipitation was performed by centrifugation of the samples at 10,000 x g for 15 min at 4 °C. The supernatant (around 250 µL) was transferred to a 1 mL NuncTM 96-well polypropylene plate (ThermoFisher), and the volume was adjusted with water to reach 1 ml (final MeOH concentration of 15%) and mixed up. SPE was then performed with a Strata-X Micro 96-well plate, 33 µm, 2 mL (Phenomenex) using a positive pressure-96 processor (Waters). After SPE plate conditioning with 2 x 0.5 mL MeOH and then 2 x 0.5 mL water, 2 x 0.5 mL of each sample were loaded on the SPE plate. After rinsing with 2 x 0.5 mL 10% MeOH in water (v/v) the analytes were eluted with 2 x 100 µL MeOH into a new 1 mL 96-well plate. Samples were transferred to a select-a-vial 96-well plate with 300 µL glass inserts (Analytical Services) and evaporated to dryness at 30 °C with nitrogen gas. Analytes were resuspended with 30 µL 50% MeOH in water (v/v), vortexed, and centrifuged for some seconds at 1000 x g before direct injection into the analytical system.

LC-MS/MS lipid mediator analysis

All samples were measured with an Exion UHPLC-system coupled to a QTRAP 6500+ mass spectrometer (SCIEX, Darmstadt, Germany) operated with Analyst 1.6.3. Chromatographic separation was achieved using a Kinetex C18 reversed phase column (1.7 μm, 100 × 2.1 mm, Phenomenex) with a SecurityGuard Ultra Cartridge C18 (Phenomenex) precolumn, heated at 40 °C. Mobile phases A, water:acetonitrile (70:30, v/v) + 100 µL acetic acid and B, acetonitrile:2-propanol (50:50, v/v) were used with gradient program with a flow rate of 500 µL/min as follow: 0% B at 0 min, 70% B at 6.5 min, 100% B at 7.8 min, 100% B at 9.5 min, and 0% B at 11 min. The autosampler was operated at 4 °C with an injection volume of 10 µL of sample.

The coupled mass spectrometer was equipped with an electrospray ionization (ESI) Turbo-VTM source set to negative mode. Source parameters were optimized to the following values: source temperature 500 °C, curtain gas flow 30 psi, ionspray voltage –4500 V, ion source gas 1 40 psi, ion source gas 2 40 psi. Metabolites were analyzed via scheduled multiple reaction monitoring (sMRM) with nitrogen as collision gas. All MRM transitions were optimized for each compound, as well as the source parameters such as declustering potential, collision energy, cell exit potential and entrance potential. The sMRM detection window was set to 60 s. Acquisition time was about 8.5 min.

SciexOS software (Sciex) was finally used for peak detection, integration and for quantitation of compounds (MQ4 algorithm). For quantification, linear calibration curves were generated from extracted calibrator samples for every compound via the IS method using the area ratio between the analyte and its ISTD, with a weighting factor of 1/x.

Data processing

Data were processed using R version 4.2.1 for the clinical study and R version 4.3.1 for cell culture experiments. A multistep procedure was used to ensure high data quality: First, lipids with a coefficient of variation of 25% in the QC-pool samples were discarded (cell culture: n= 4, clinical study: n = 4). We then discarded lipids where missing values (NA) were observed in more than 50% per treatment group in all investigated treatment groups (cell culture: n = 25, clinical study: n = 11). Missing values (cell culture: n = 0, clinical study: n = 211; 6.8% of whole data set) were imputed using the k-nearest-neighbour obs-sel imputation approach as described by Do *et al.* using k = 3 [4].

**Supplemental results:**

RNAseq revealed consistent expression of *CYP1A1* in endothelial cells under all treatment conditions. Only treatment with GSK, aldo and cortisol resulted in a significant reduction in expression levels (Supplemental figure 1).

Ein Bild, das Text, Screenshot, Diagramm, Zahl enthält.

Automatisch generierte Beschreibung

**Supplemental Figure 1**: Expression levels of CYP1A1 as potential EET synthesizing epoxigenase in HCAEC. Read counts for CYP1A1 were normalized to the geometric mean of read counts for EIF2B1 and HPRT1. Horizontal bars indicate median values. Aldo, aldosterone, Ep, eplerenone, GSK, GSK2256294. \*\*\*, p<0.001, one-way ANOVA, Holm-Šídák.

We could not reproduce a relationship between plasm aldosterone concentrations and the sum of all detected EET in the clinical cohort. **Supplemental Figure 2** shows the pooled cohort. Separate assessment of both subgroups (EH and PA) neither revealed a significant relationship.



**Supplemental Figure 2**: No apparent relationship between plasma aldosterone concentrations and the sum of all detected EET. Both groups were pooled together with the blue dots representing the EH patients and the red dots the PA patients. Black line indicates linear regression line; EH, essential hypertension; PA, primary aldosteronism; P=0.9599 (slope being different to non-zero).

**References**

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