SUPPLEMENTARY MATERIAL

A Prospective Cohort Study in Patients with Type 2 Diabetes Mellitus For Validation of Biomarkers (PROVALID) – Study Design and Baseline Characteristics

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Supplementary Table 1:

PROVALID BASELINE CLINICAL PARAMETER LIST: DATA COMPLETENESS

Parameter	% completeness of data
Patient identifier	100
Date of visit	100
Year of birth	100
Race	100
Gender	100
Body weight	96.4
Height	91.9
Blood pressure	98.6
Year of diabetes type 2 diagnosis	96.8
Year of start of antidiabetic pharmacological treatment	84.7
Year of diagnosis of hypertension	62.2
Year of start of antihypertensive pharmacological treatment	60.9
Diabetic retinopathy	97.9
Renal disease	97.9
Heart failure NYHA III or IV	95.7
Coronary artery disease	97.8
Peripheral artery disease	97.7
Cerebrovascular disease	97.7
Smoking	77.1
Malignancy	97.4
Medication	97.0
Blood glucose	95.3
HbA1C	98.1
Serum creatinine	98.9
Urinary albumin excretion	96.4
Urinary creatinine excretion	96.7
Diagnosis of albuminuria	96.4

Supplementary Table 2:

SOPs FOR BASELINE SAMPLE COLLECTION IN PROVALID

Sampling materials per patient	1x Urine-container 100 ml (Sarstedt)
	1x Urine-container lid (Sarstedt)
	3x Urine-Monovettes 10 ml (Sarstedt)
	Butterfly canula (Sarstedt)
	1x S-Monovette K-EDTA 1.2 ml (Sarstedt)
	1x S-Monovette K-EDTA 4 ml (Sarstedt)
	1x S-Monovette K-EDTA 4.9 ml (Sarstedt)
	2x S-Monovettes K-EDTA 9 ml (Sarstedt)
	1x S-Monovette SERUM 1.2 ml (Sarstedt)
	1x S-Monovette LiHeparin 4.9 ml (Sarstedt)
	2x PAXgene blood RNA tubes 2.5 ml (Quiagen)
	1x PAXgene blood DNA tube 8.5 ml (BD Biosciences)
	32x PCR-clean Safe-Lock micro test tubes 2 ml (Eppendorf)
	3x 15 ml Falcon tubes (BD Biosciences)
Equipment	Centrifuge for sample preparation
	Gilson Pipettes P20, P200, P1000 and pipette tips in boxes
	Styrofoam box with dry ice
Reagents	Butylhydroytoluol (0,001%) - BHT (Sigma-Aldrich): antioxidans for plasma metabolomics
	Protease inhibitor-mixture for urinary exosome analysis: 100 mM aqueous sodium azide (Sigma-Aldrich), stable at room temperature
	2 mg/ml Phenylmethylsulfonylfluorid - PMSF (Sigma-Aldrich) in Isopropanol (Sigma Aldrich), stable at +4°C for several months
	Leupeptin (Sigma-Aldrich): 1 mg/ml in ddH2O, stable for 1 week at +4°C, 6 months at -20°C
	Thermo Scientific Halt Protease Inhibitor-Cocktail, EDTA free 100x (Fisher Scientific)

Procedure

Patient condition: fasting for > 12 hours

Case report form (CRF): completion of appropriate baseline documents

Sample labelling: for each patient a unique label sheet is provided at each recruiting site

Collection volume per patient: 47.7 ml venous blood and 25 ml second morning mid-stream urine. If a first morning void is used this needs to be documented.

All blood and urine samples have to be collected on the same day and at the same visit. It is essential that there is thorough documentation of how the samples were handled (to be noted in the lab book). Reproducibility is important; all samples from different patients should be prepared as similar as possible.

Minimal required information for all collected samples (to be noted in the lab book):

1. Unique Sample Code (label to be taken from the prepared label sheet)

2. Date and time of collection

3. Volume) and number of aliquots

4. Date and time of shipment (samples have to be shipped on dry ice on the collection day to the national storage site)

The whole collection and processing material of each patient has to be labelled properly. All actions not performed according to the protocol have to be noted in the lab book.

Blood:

Butterfly cannula and labeled blood collection tubes are prepared by the technician.

1x S-Monovette K-EDTA 1.2 ml

1x S-Monovette K-EDTA 4 ml

1x S-Monovette K-EDTA 4.9 ml

2x S-Monovettes K-EDTA 9 ml

1x S-Monovette SERUM 1.2 ml

1x S-Monovette Li-Heparin 4.9 ml

2x PAXgene blood RNA tubes 2.5 ml

1x PAXgene blood DNA tube 8.5 ml

15x PCR-clean Safe-Lock micro test tubes 2 ml

Venous blood is drawn by the physician into the tubes and prepared for further processing.

Blood preparation:

1.2 ml K-EDTA Monovette: Store EDTA blood at +4°C until shipment on dry ice. Use the appropriate label from the label sheet. Mark the vial with “routine analysis EDTA blood” with a waterproof pen.

4 ml K-EDTA Monovette: Store EDTA blood at -20°C until shipment on dry ice. Use the appropriate label from the label sheet. Mark the vial with “SNP EDTA blood” with a waterproof pen.

4.9 ml K-EDTA Monovette: Shake properly. If centrifugation is not done immediately put them in the freezer (+4°C). Centrifuge at 3000 rpm for 10 min. Pipette 4 aliquots (0.5 ml each) of the EDTA plasma into 2 ml micro test tubes. Add the antioxidant BHT to two vials (final concentration 0.01µl/ml). Use the appropriate labels from the label sheet. Mark two vials with “metabolomics plasma + BHT” and two vials with “metabolomics plasma” by using a waterproof pen. Store tubes at +4°C until shipping on dry ice.

9 ml K-EDTA Monovette: Shake properly, leave the tube for 20 min at RT, centrifuge immediately after these 20 min at 3000 rpm for 10 min. Pipette the supernatant into four fresh 2 ml micro test tubes (1 ml each). Use the appropriate labels from the label sheet. Mark the vials with “CE-MS analysis plasma” by using a waterproof pen. Store tubes at +4°C until shipping on dry ice.

9 ml K-EDTA Monovette: Shake properly, leave the tube for 20 min at RT, centrifuge at 3000 rpm for 10 min. Transfer the supernatant into four 2 ml micro test tubes (1 ml each). Add Thermo Scientific Protease Inhibitor-Cocktail, EDTA free (100x) to two of the viles (10µl/ml plasma). Use the appropriate labels from the label sheet. Mark two vials with “biomarker analysis + Protease Inhibitor” and two with “biomarker analysis - Protease Inhibitor” by using a waterproof pen. Store at -20°C before shipment on dry ice.

1.2 ml SERUM Monovette: Shake properly, leave the tube for 10-15 min at RT, centrifuge at 3000 rpm for 10 min. Pipette 0.5 ml of the supernatant into a fresh 2 ml micro test tube, use the appropriate label from the label sheet. Mark the vial with “routine analysis serum” by using a waterproof pen and store at +4°C until shipment on dry ice.

4.9 ml Li-Heparin Monovette: Shake properly, leave the tube at RT for 10-15 min , centrifuge at 3000 rpm for 10 min. Pipette 1.8 ml of the supernatant into a fresh 2 ml micro test tube, use the appropriate label from the label sheet and mark the vial with “routine analysis heparin plasma” by using a waterproof pen. Store the heparinized plasma at at +4°C until shipment on dry ice.

2x 2.5 ml PAXgene blood RNA tubes: Use the appropriate labels from the label sheet; mark the two tubes with “miRNA analysis blood” with a waterproof pen. Refrigerate at –20°C, ship on dry ice.

8.5 ml PAXgene blood DNA tube: Use the appropriate label from the label sheet, mark the tube with “epigenetic analysis blood” by using a waterproof pen. Freeze at –20°C, ship on dry ice.

Urine:

Labelled urine collection tubes are prepared by the technician.

3x Urine-Monovettes 10 ml

1x Urine-container 100 ml

1x Urine-container lid

3x 15 ml Falcon tubes

17x PCR-clean Safe-Lock micro test tubes 2 ml

Urine is collected in the urine container and covered with a lid.

Urine preparation:

Pipette 1 ml of the urine into a 2 ml micro test tube, centrifuge at 3000 rpm for 10 min, pipette supernatant into a fresh 2 ml micro test tube. Use the appropriate label from the label sheet and mark the vial with “routine analysis urine” by using a waterproof pen. Store at +4°C until shipment on dry ice.

Use a 10 ml Urine-Monovette to transfer 10 ml of the urine into a 15 ml Falcon tube, add the protease inhibitor solution (volume per 10 ml urine): 334 µl of 100 mM aqueous sodium azide, 500 µl PMSF solution (2 mg/ml in isopropanol) and 10 µl Leupeptin solution (1 mg/ml in ddH2O). Prepare six aliquots containing 1.5 ml each by using 2 ml micro test tubes. Use the appropriate labels from the label sheet and mark all the vials with “urinary exosome analysis” by using a waterproof pen. Refrigerate at -20°C, ship one dry ice.

Fill another Urine-Monovette with 10 ml urine from the Urine-container. In general, samples should not be turbid. Spin if haemorrhagic or cellular contents are obvious (15 min, 2000 rpm) and transfer the liquid into a fresh 15 ml Falcon tube. Prepare six aliquots of 1.5 ml each in 2 ml micro test tubes. However, centrifuged samples may result in data of lower quality. Make an appropriate mark on these samples if centrifuged and report this in the lab book. Use the appropriate labels from the label sheet and mark the vials with “CE-MS analysis urine” by using a waterproof pen. Store at +4°C until shipping on dry ice.

Fill another Urine-Monovette with 3 ml urine. After centrifugation for 15 minutes at 2000 rpm the supernatant should be transferred within one hour into a 15 ml Falcon tube. Prepare four aliquots by using 2 ml micro test tubes, containing 600 µl each. Samples have to be stored at -20°C within 2 hours after collection. Use the appropriate labels from the label sheet. Mark the vials with “metabolomics urine” by using a waterproof pen. Ship on dry ice.