**Protocol for the combined cardiometabolic deep phenotyping and registry-based twenty-year follow-up study of the Inter99 cohort.**

## SUPPLEMENTAL METHODS

### Deep phenotyping follow-up study

*Arterial stiffness*

Arterial stiffness will be assessed based on carotid-femoral Pulse Wave Velocity (cfPWV) a non-invasive measure considered the gold standard method of assessing direct arterial stiffness [1]. The SphygmoCor XCEL instrument (AtCor Medical, Sydney, Australia) will be applied to measure cfPWV. cfPWV measurements are performed under standardized conditions according to guidelines [1] and follow the quality demands suggested by the manufacturer. Prior to the measurement, the participant must be fasting for 3 hours (including the absence of coffee, tea, smoking, and alcohol) and resting in a lying position for 10 minutes in a quiet room. Blood pressure is measured three times with a Microlife BP A6 PC blood pressure device, and the mean blood pressure is used. cfPWV is defined as the distance between the two recording sites divided by the difference in pulse wave travel time and expressed in meters per second. Distance is directly measured as a straight line by a caliper from the recording sites at the carotid to the femoral artery, and the total distance is multiplied by 0.8 [1]. The transit time is based on measurements of pulse waves assessed by use of an applanation tonometer at the carotid artery on the neck and from a blood pressure cuff on the thigh. cfPWV measurements will be performed twice, and if these vary by more than 0.5 m/s, a third measurement will be performed.

*Biochemistry*

- *B-Leucocytes, B-Platelets and B-Leucocytes*: differential count will be sampled in EDTA-stabilized blood and analyzed on the Sysmex XN (Sysmex Corporation, Kobe, Japan) analyzer.

- *P-Sodium and P-Potassium*: will be sampled in Li-heparin-containing tubes and centrifuged to separate plasma and subsequently analyzed by potentiometric slide test on Vitros 4600/5600 instruments (QuidelOrtho, Raritan, USA).

- *P-Calcium (total)*: will be measured in Li-heparin plasma using a colorimetric slide test on Vitros 4600/5600 instruments.

- *P-Glucose*: blood will be collected in citrate buffer-fluoride mixture (FC-Mixture) tubes and analyzed on Vitros 4600/5600 instrumnents (QuidelOrtho) and P-HbA1c measurements will be done using EDTA-stabilized blood using a HPLC-based method on the Tosoh G8 instrument (Tosoh Bioscience, San Francisco, USA).

- *P-Total cholesterol, P-Triglyceride, and P-High-density lipoprotein (HDL) cholesterol*: blood will be collected in Li-heparin tubes and centrifuged and measured using colorimetric slide tests on the Vitros 4600/5600 instruments (QuidelOrtho). P-Very-low density lipoprotein (VLDL) cholesterol and P-Low-density lipoprotein (LDL) cholesterol will be calculated from the formula P-VLDL = 0.45 * P-Triglyceride; P-LDL = P-Total Cholesterol - HDL - VLDL.

- *P-Alanine amino transferase (ALT), P-Aspartate aminotransferase (AST), P-Creatinine,P-Albumin and P-Urea*: blood will be collected in Li-heparin tubes, centrifuged and analytes will be measured using colorimetric slide tests on the Vitros 4600/5600 (QuidelOrtho). Estimated Glomerular Filtration Rate (eGFR) will be calculated using the CKD-EPI formula.

- *P-Aldosterone and P-Renin*: blood will be sampled in EDTA-containing tubes, centrifuged and plasma stored at -80°C until analysis. Measurements will be done using chemiluminescence immunoassays on the dedicated IDS iSYS instrument (IDS PLC, Tyne and Wear, UK).

- *P-Dephosphorylated-uncarboxylated matrix-gla Protein (MGP):* blood will be sampled in EDTA-containing tubes, centrifuged and plasma stored at -80°C until analysis using the InaKtif MGP assay, which is a chemiluminescence immunoassay, on the IDS iSYS instrument (IDS PLC).

- *P-Aldosterone, P-Renin and P-MGP*: will be analyzed in one single batch to reduce variability on the measurements. All other analyses will be measured right after arrival at the clinical biochemistry laboratory.

*Cardiac Computed Tomography (CT) scans*

Cardiac CT scans will include a non-contrast CT scan to evaluate CAC score, aortic valve calcifications, lung density analysis, and bone mineral density (BMD). Furthermore, a CT angiography is applied to evaluate cardiovascular and heart structures and subclinical obstructive coronary atherosclerosis. In addition to cardiac risk assessment, the CT scan includes comprehensive imaging of lungs, vascular system, sarcopenia assessment, liver, spleen, abdominal fat, and spine.

CT imaging will be performed using a 320-multidetector scanner (Aquilion One, Toshiba Medical Systems). Participants are instructed to abstain from coffee, tea, cocoa, and chocolate from 4 p.m. the day before the CT scan. For participants with a heart rate of >60 bpm and no contraindications, a cardio-selective beta-blocker (metoprolol 25–150 mg) is administered orally prior to the CT scan. Intravenous contrast media (Visipaque) is given after assessment of kidney function (estimated Glomerular Filtration Rate (eGFR) >60 ml/min/1.73m2). A protocol using one rotation acquisition will be used. The total dose of radiation received from a single cardiac CT scan is approximately 3–10 mSv. For comparison, the average annual limit for radiation workers is 20 mSv and Denmark's annual background radiation dose is 3 mSv. According to the Danish National Committee on Biomedical Research Ethics, a radiation dose of 10 mSv may increase cancer risk by 0.05 % [1].

*Muscle Strength (hand grip and Sit-to-Stand test)*
The participant will be sitting in an upright position with the arm along the side; and the arm bent at 90° with the elbow, forearm, and wrist in a neutral position. The width of the handle will be adjusted to fit the hand size. Hand grip will be measured three times in the dominant hand with brief pauses between each measurement and the best three measurements considered as the maximum hand grip strength [5]. Verbal instructions will be given before performing the Sit-to-Stand test (STS) test. After the cue "ready set, go!" the participant will start to do STS repetitions as rapidly as possible from the sitting position, with arms crossed over the chest. Participant will perform the test five times, and the time needed to complete the task will be recorded with a stopwatch to the nearest 0.01 s. The subjects will be allowed to try 1-2 times with a resting period (30-60 s) before the definitive STS measure is annotated [6].

**Extended clinical sub-studies nested within the twenty-year follow-up of the Inter99 cohort**

***1. The InterVitaminK trial***
As part of the InterVitaminK trial lung function will be assessed, Hereby, longitudinal spirometry data will be available in a sub-sample of the Inter99 20-year follow-up study [7].

*Spirometry*
Pulmary function will be measured through spirometry performed with Vyntus SPIRO (Vyaire Medical), disposable MicroGard pulmonary function filters with nose clips (V-892391) and Sentrysuite software (V3.20.3). The examinations will be performed according to the 2005 American Thoracic Society and the European Respiratory Society (ATS/ERS) spirometry standard [8] after a daily calibration with a 3-litre calibrated syringe. The spirometer calibration syringe will be calibrated yearly to comply with the international standard [9]. Body weight is measured using a digital scale (Tanita, BC 420), and 1 kg is automatically subtracted to account for the weight of the participant's clothes. Height is measured without shoes with a Holtain Harpenden Stadiometer (model: 602VR). Respiratory function measurements, i.e., expiratory forced vital capacity (FVC) and forced expiratory volume in one second (FEV1), will be conducted.

***2. The InterDAG Study***
Participants will receive information about and be invited to participate in ten days continuous glucose monitoring (CGM) and physical activity accelerometer measurements as well as seven-day food registration, three day 24-hr urine collection and one stool collection. Participants will be instructed to follow their usual routines during the collection period.

*Ten-day Physical activity measurements by Sens Motion® accelerometers*
Physical activity and sedentary behavior will be monitored by continuous 24-hour*10 days measurement using Sens Motion® accelerometers (www.sens.dk) skin-taped to the right thigh. It will be possible to classify behavior second-by-second into the following activity types: sitting/lying,

standing, walking, running, and cycling. Raw accelerometer data will also be classified as time spent in different intensity levels, including vigorous, moderate, and light intensity activity. The Sens Motion® accelerometer collects second by second movement data from three axes (vertical, horizontal, and lateral), and is small, (45 x 4.5 x 23 mm) lightweight 7g) and waterproof with a sampling acceleration at 12 Hz and a range of ±4G. The Sens Motion patch will be attached to the thigh using hypo-allergenic dressing at the CCRP. After wearing the accelerometer for 24-hours*10 days, participants will return the accelerometer to the CCRP and collected data will be automatically transmitted to secure Cloud storage via the smartphone app. During the 10 days of wearing the Sens Motion® accelerometer, participants will be asked to keep a log on daily work and sleep times.

*Seven-day food record*

At the end of each day, participants will register food and drink intake, applying the online dietary assessment software myfood24®. It is structured according to a typical meal pattern covering breakfast, lunch, dinner and snacks plus drinks. The participants will be able to search for items and estimate the consumed amount by selecting the closest portion size using portion size pictures, provided weights, or entering an exact amount. Internal prompts for frequently forgotten items like condiments, snacks, confectionary, and beverages are included. And there is also a recipe builder feature. Furthermore, there is the option for participants to report intake of nutritional supplements and if the day represented a usual or unusual intake, including reasons for unusual intakes such as illness or special occasions. To assist recordings, participants will be given a 7-day food diary to record food intake. If the needed computer skills are lacking, the paper food diary will be recorded by the staff.

*Three-day 24 hr urine sample*

The urine collection will be carried out simultaneous with the 7-day food record. Participants will receive a brown bottle (3 L), and a smaller 'visiting bottle' (0.5 L), a large bottle and urine monovettes (Sarstedt, Nümbrecht-Rommelsdorf, Germany) for collection of urine aliquots after the completion of each 24 h collection period. A pen to mark containers and monovettes with name, day, and volume. For validation participants will also receive 3 times 3 80 mg para-aminobenzoic acid (PABA) tablets (Glostrup Hospital Pharmacy). A sheet to register beginning and ending of the collection periods, PABA administration and exceptions to the protocol (i.e., estimation of urine loss, medicine). Participants will be informed to collect 24 h urine for three consecutive days (1 weekend day, 2 working days). All participants will receive verbal and printed instructions (including a video link) on how to collect 24 h urine: All urine must be collected during a 24 h period starting from the second urine sample on the morning of the collection day and ending with the first urine sample from the following morning. The morning, after completion of the 24 h urine collection participants must mark the volume and day of the collection on the container and registered values in the data sheet. Also, the time of start and finish of the urine collections, and the time of taking the PABA tablets will be recorded together with deviations to the instructions. After volume recording, the urine in the container will be mixed before taking out aliquots. Hereafter monovettes will be frozen at home -20 °C until returned to CCRP. Containers will be rinsed with water and participants can resume their next 24 h urine collection.

A well-trained health worker will check the readings of the total volume marked on the containers and urine aliquots will stored at -80 °C before being transported to a certified laboratory for analysis of sodium, potassium, albumin, creatinine and for PABA analyses. Based on the participants daily recordings of diuresis the 24 h-values of sodium, potassium, albumin and creatinine will be determined. PABA is an accepted objective marker to verify completeness of 24 h urine sampling in adults [10]. The underlying assumption is that PABA is excreted almost quantitatively in 24 h. On collection days adults ingested 240 mg of PABA, divided into three doses of 80 mg (one with each main meal). According to the HPLC method applied a PABA recovery in the urine above 77.9% of total ingested dose indicates urine has been collected for 24 h [11]. However, PABA recovery levels above 105% will be regarded as mistaken. If PABA recovery is not available urine collections with collection time less than 22.5 h or more than 25.5 h will be excluded as well as urine collections with volume <500 mL/24 h for adults [11].

*Fecal sample*

Stool samples will be collected at home by the participants in a 5 mL tube, and directly put in minus 20 freezers. Samples are transported from home to the lab in an insulated bag and stored in a minus 80 freezers. At site the stool samples are aliquoted by the MGISTP-7000 robot to a 96well format. The DNA extraction itself takes place in MGISTP-960well robot, using the MGIEasy Stool Microbiome DNA extraction kit and its buffers (Cat.no 940-000122-00, MGI). Sequencing is done in the DNBSEQ-G400 from MGI using HotMPS High-throughput Sequencing Set (Cat.no 940-000091-00, MGI) for library preparations with a depth of 10GB/sample. Protocols written by the manufacture will be followed.

**Deviations between examinations at baseline and twenty-year follow-up of the Inter99 cohort**

- Participants were fasting from 11 pm the night before baseline examinations compared to a minimum of six hours before examinations at follow-up.
- Blood was drawn from a peripheral venous catheter as well as a capillary sample taken from the finger or earlobe at baseline examinations as opposed to vacuettes used for blood sampling at follow-up.
- Spot urine samples were collected throughout the day, as compared to morning spot urine collection at follow-up.
- Blood pressure was measured by a mercury manometer at baseline and by an electronic blood pressure monitor at follow-up. At baseline the third measurement was only performed if blood pressure was above 140 systolic or 90 diastolic. At follow-up blood pressure is measured three times one minute apart.
- At Inter99 baseline ECGs were recorded using the Cardiosoft system GE Healthcare, Milwaukee, WI, USA while the devise used to record the ECGs at 20-year follow-up was a GE MAC VU 360.
- Ophthalmic examination a baseline included a 7-field non-stereoscopic 60-degree digital fundus photography (TRC-50X camera; Topcon, Tokyo, Japan) [12] and a follow-up ocular

wide-field fundus photography and optical coherence tomography (OCT) are made using the Optos Monaco device (Optos PLC, Dunfermline, UK) [13].

- Deep phenotyping examinations introduced at twenty-year follow-up study were:
  - Arterial stiffness
  - Body composition measured by impedance
  - Cardiac autonomic neuropathy
  - Coronary artery calcification
  - Fundus characteristics measured by OPTOS scanning
  - Liver stiffness and steatosis
  - Muscle strength
  - Oxygen saturation

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