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Complete List of Authors:	Bjørnsbo, Kirsten; Frederiksberg Hospital Center for Klinisk Forskning og Forebyggelse Brøns, Charlotte; Steno Diabetes Center Copenhagen, Aadahl, Mette; Frederiksberg Hospital Center for Klinisk Forskning og Forebyggelse Kampmann, Freja; Frederiksberg Hospital Center for Klinisk Forskning og Forebyggelse Friis Bryde Nielsen , Camilla; Frederiksberg Hospital Center for Klinisk Forskning og Forebyggelse Lundbergh, Bjørn; Frederiksberg Hospital Center for Klinisk Forskning og Forebyggelse Christensen, Rasmus; Steno Diabetes Center Copenhagen Kårhus, Line Lund; Frederiksberg Hospital Center for Klinisk Forskning og Forebyggelse, Section of Epidemiology Madsen, Anja; Frederiksberg Hospital Center for Klinisk Forskning og Forebyggelse Hansen, CS; Steno Diabetes Center Copenhagen Nørgaard, Kirsten; Steno Diabetes Center Copenhagen Jørgensen, Niklas; Rigshospitalet, Clinical biochemistry Suetta, Charlotte; University of Copenhagen, Copenhagen University Hospital, Bispebjerg and Frederiksberg Kjaer, Michael; University of Copenhagen, Department of Clinical Medicine, Grarup, Niels ; University of Copenhagen Faculty of Health and Medical Sciences, Novo Nordisk Foundation Center for Basic Metabolic Research Kanters, Jørgen; University of Copenhagen Novo Nordisk Foundation Center for Basic Metabolic Research Larsen, Michael; University of Copenhagen, Køber, Lars; Rigshospitalet, Department of Cardiology Kofoed, KF; University of Copenhagen, Cardiology, section 2014 Loos, Ruth; University of Copenhagen Novo Nordisk Foundation Center for Basic Metabolic Research, Copenhagen University Hospital, Bispebjerg and Frederiksberg Hansen, Torben; University of Copenhagen Novo Nordisk Foundation Center for Basic Metabolic Research, Center for Basic Metabolic Research, Section of Metabolic Genetics linneberg, allan; Frederiksberg Hospital Center for Klinisk Forskning og Forebyggelse, Research Centre for Prevention and Health Vaag, Allan; Steno Diabetes Center Copenhagen

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

Kirsten Bjørnsbo[1*], Charlotte Brøns[2*], Mette Aadahl[1], Freja Bach Kampmann[1], Camilla Friis Bryde Nielsen[1], Bjørn Lundbergh[1], Rasmus Wibaek[2], Line Kårhus[1], Anja Lykke Madsen[1], Christian Stevns[2], Kirsten Nørgaard[2,3], Niklas Rye Jørgensen[4], Charlotte Suetta[5], Michael Kjær[5], Niels Grarup[6], Jørgen K. Kanters[4,7], Michael Larsen[3,8], Lars Køber[9], Klaus F. Kofoed[3,9], Ruth J.F. Loos[6], Torben Hansen[6], Allan Linneberg[1,3**], Allan Vaag[2,10**]

* Contributed equally to this work, ** Shared last authorship

[1] Center for Clinical Research and Prevention, Copenhagen University Hospital – Bispebjerg and Frederiksberg, Copenhagen, Denmark
[2] Steno Diabetes Center Copenhagen, Herlev, Denmark
[3] Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
[4] Department of Clinical Biochemistry, Copenhagen University Hospital – Rigshospitalet, Copenhagen, Denmark
[5] Institute of Sports Medicine, Dept of Orthopedic Surgery and Department of Geriatrics and Palliative Medicine, Copenhagen University Hospital – Bispebjerg and Frederiksberg, Copenhagen, Denmark
[6] Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[7] Center of Physiological Research, University of California San Francisco, San Francisco, CA, USA
[8] Department of Ophthalmology, Copenhagen University Hospital – Rigshospitalet, Glostrup, Denmark
[9] Department of Cardiology and Radiology, Copenhagen University Hospital – Rigshospitalet, Copenhagen, Denmark
[10] Lund University Diabetes Centre, Malmö, Sweden

Corresponding authors: Kirsten Bjørnsbo kirsten.bjoernsbo@regionh.dk and Charlotte Brøns charlotte.broens.01@regionh.dk

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Abstract

Introduction The population-based Inter99 cohort has contributed extensively to our understanding of effects of a systematic screening and lifestyle intervention, as well as the multifactorial etiology of type 2 diabetes (T2D) and cardiovascular disease (CVD). To understand courses, trajectories, and patterns of early and overt cardiometabolic disease manifestations, we will perform a combined clinical deep phenotyping and registry follow-up study of the now 50-80 years old Inter99 participants

Methods and analysis The Inter99 cohort comprise individuals aged 30-60 years, who lived in a representative geographical area of greater Copenhagen, Denmark, in 1999. Age- and sex-stratified random subgroups were invited to participate in either a lifestyle intervention (N=13,016) or paper surveys (N=5,264), while the rest served as a reference population (N=43,021). Of the 13,016 individuals assigned to the lifestyle intervention group, 6,784 (52%) accepted participation in a baseline health examination in 1999, including screening for cardiovascular risk factors and prediabetic conditions. All eligible participants will be invited for a deep phenotyping 20-year follow-up clinical examination including measurements of anthropometry, blood pressure, arterial stiffness, cardiometabolic biomarkers, coronary artery calcification, heart rate variability, heart rhythm, liver stiffness, fundus characteristics, muscle strength and mass, as well as health and lifestyle questionnaires. In a subsample, 10-day monitoring of diet, physical activity and continuous glucose measurements will be performed. Fasting blood, urine, and fecal samples to be stored in a biobank.

Ethics and dissemination The study was approved by the Medical Ethics Committee, Capital Region, Denmark (H-20076231) and by the Danish Data Protection Agency through the Capital Region of Denmark's registration system (journal number P-2020-1074). Findings will be disseminated in peer-reviewed journals, at conferences, and via presentations to stakeholders, public and policymakers. Data may become available for international collaborations upon request.

ClinicalTrials.gov registration number NCT05166447.

Strengths and limitations of this study

1. The longitudinal design will enable us to follow the course of both early and overt cardiometabolic disease manifestations during the period of life with highest incidence rates.

2. We will be able to quantify the extent to which the T2D associated co-morbidities coronary arteriosclerosis, cardiac autonomic neuropathy, non-alcoholic fatty liver disease (NAFLD), retinopathy, and diabetic kidney disease are present among elderly people without T2D, and with known normal glucose tolerance for two decades.

3. The availability of genome-wide genetic variation data, birthweight, as well as adiposity trajectories, dietary data, biomarkers of micronutrient status, and physical activity information, will provide insights into how these predisposing factors influence distinct organ morbidities and disease manifestations.

4. Collection of biospecimens for micronutrient and multi-omics purposes such as genomics, transcriptomics, proteomics, metabolomics, lipidomics, and epigenomics will allow additional layers of deep phenotype analyses, including studying disease-associated genetic variants and their phenotypes.

5. Limitations include the observational nature of the study precluding causality inferences. The fact that all individuals participated in a personalized lifestyle intervention from 1999 and for up to five years thereafter, may limit the generalizability of our findings.

Box 1: The complex multifactorial pre- and postnatal etiology of cardiometabolic diseases

Constitutional primary predisposing factors
- Genetic susceptibility
- Intrauterine environment (low or high birth weight, prematurity)

Acquired postnatal secondary precipitating factors
- Sedentary lifestyle / inactivity
- Unhealthy diet / micronutrient deficiencies
- Obesity
- Smoking
- Medication
- Comorbidities
- Aging

Introduction

Type 2 diabetes (T2D), cardiovascular disease (CVD) and their co-morbidities are leading causes of premature mortality and morbidity, affecting nearly one billion individuals worldwide [1,2]. T2D is arbitrarily defined by elevations of plasma glucose levels, and the current T2D diagnostic criteria does not capture the diversity of T2D sub-phenotypes characterized by differential manifestations of micro - and macrovascular complications, as well as other common comorbidities [3,4].

The overlap and heterogeneity of age-related T2D, CVD and associated comorbidities are likely rooted in the relative or predominant contributions from the triad of genetic susceptibility versus pre- and postnatal non-genetic etiologies (**Box 1**). As for genetics, the known 568 T2D susceptibility loci are estimated to explain 18% of the putative genetic contribution to T2D [5,6]. Early life developmental programming, low birth weight (LBW), as well as salt-sensitive hypertension, non-alcoholic fatty acid disease (NAFLD), dyslipidemia, and neurocognitive dysfunctions, are well-established risk factors of T2D and CVD [7–12]. Recent data even suggest that LBW, in a non-genetic manner, is associated with a more severe clinical T2D presentation and course, including earlier onset and more comorbidities at the time of diagnosis [13]. Accordingly, there is an increasing need to understand whether differential combinations of T2D etiologies influence not only T2D and CVD risk *per se*, but also the timing and patterns of clinical presentation including both early and late disease manifestations, as well as co-morbidities. As an example of unprioritized co-morbidities, T2D patients have a two-three-fold increased risk of sarcopenia [14,15]. Sarcopenia describes the age-related loss of muscle mass and strength that leads to impaired function including increased risk of falls and an overall decreased quality of life. Sarcopenia is accelerated by physical inactivity, low protein intake, and general health status and disease, and has also been associated with LBW [16].

Excess dietary sodium (Na^+) may account for three million deaths annually [17], and reducing salt intake is among the most cost-effective CVD prevention strategies [18]. Low dietary potassium (K^+) intake is also gaining attention as a CVD risk factor, and the urinary Na^+/K^+-ratio may therefore represent a superior cardiovascular risk measure [19–23]. As for macronutrient intake, high dietary sugar and saturated fat contents is strongly associated with T2D and CVD risk. While the Mediterranean diet may prevent CVD [24], there is nevertheless substantial gaps in our current knowledge of what defines a healthy diet with respect to not only macro but also micro nutritional composition(s) including vitamins. For instance, beyond effects on blood clotting factors, vitamin K may be important for cardiometabolic as well as bone health [25].

The Inter99 cohort provides a unique research platform to delineate the differential and overlapping roles of genetics versus the fetal environmental, as well as various postnatal lifestyle factors, for the development of early and overt cardiometabolic disease manifestations and associated co-morbidities [26–28]. We aim to perform a combined deep phenotyping and registry-based follow-up study of the Inter99 cohort, 20 years after the baseline health examinations, when participants were on average 46 years of age. While overt disease diagnoses will be captured by Danish national registries, our deep phenotyping clinical examinations allow detection of a wider range of early disease manifestations, such as vascular stiffness, liver fibrosis, retinopathy, diabetic kidney disease, and prediabetes, and the

extent to which these may be present prior to the participants complying with official cardiometabolic disease diagnoses.

Methods and analysis

Study setting and previous findings

The Inter99 study, initiated in March 1999, is a population-based multi-factorial intervention study, originally designed to prevent ischemic heart disease (IHD) [29]. It comprised all individuals born in 1939-40, 1944-45, 1949-50, 1954-55, 1959-60, 1964-65, and 1969-70 (30, 35, 40, 45, 50, 55, and 60 years of age) living in 11 municipalities in Greater Copenhagen (N=61,301).

The population was randomized with different age and sex ratios to two lifestyle intervention groups (A+B; N=13,016) or a group followed by questionnaires (C; N=5,264), the remaining individuals were considered as a reference population and not contacted (D; N=43,021) (**Figure 1**). Participants received individualized lifestyle counseling based on lifestyle and CVD risk score [30]. In total, 6,784 participants from the intervention groups participated in the baseline health examination (52% acceptance rate) [29]. The 5-year clinical follow-up examination, including glucose tolerance status, had a participation rate of 69% [31]. The 10-year follow-up was based on registry data and a follow-up questionnaire was sent to all eligible participants with completed baseline health examination (A+B) and to all in the paper survey group (C) (**Figure 1**) [32].

The 5-year follow-up examination showed a progression rate to overt T2D of 2.1 per 100 person-years [33], strongly supported by a substantially higher T2D prevalence after 20 years of follow-up with 6.5% of men and 3.8% in women having been diagnosed with T2D. Indeed, this will also be the case for all other T2D-associated vascular and cardiometabolic co-morbidities, underscoring the relevance of performing a combined registry-based and cardiometabolic deep phenotyping study among Inter99 participants, who are now 50-80 years old.

Original midwife records were collected from 4,744 participants in the intervention groups [34,35], and despite the relatively low average age of 46 years in 1999, we confirmed a strong inverse relationship between birthweight and risk of T2D in this Danish population [35]. The Inter99 cohort has been extensively genotyped contributing to the identification of more than 568 T2D susceptibility loci [5], and to the interactions between birthweight and genetic risk of T2D [27,28,36]. The prevalence of T2D associated retinopathy in the Inter99 cohort was studied in a subgroup of 970 participants. Interestingly, retinopathy was present in 7.5% of the 490 subjects with completely normal glucose tolerance, supporting the notion that factors other than elevated glucose contributes to the risk of retinopathy of a type that cannot be distinguished from milder degrees of diabetic retinopathy [37].

The 10-year follow-up study concluded that a community-based, individually tailored intervention program with screening for risk of ischemic heart disease and repeated lifestyle intervention over 5 years, had no effect on ischemic heart disease, stroke, or mortality at the population level as assessed after 10 years [38]. This observation was later reproduced in other studies and confirmed in a WHO report [39].

The totality of data already available in the Inter99 cohort range from information on birth size (weight and length) and prematurity, glucose tolerance at baseline and at 5-year follow-up, lifestyle intervention and general health information including comprehensive dietary data, numerous biomarkers, and genetic data (**Table 1, Figure 2**). The 20-year follow-up study includes physical deep phenotyping clinical examinations tailored to capture and expand our growing understanding of T2D and subgroups, as well as early and late metabolic and vascular manifestations, co-morbidities, and complications. Morbidity and mortality data is obtained from our extensive Danish registers. The existing detailed clinical and lifestyle information over 20 years will allow us to correct for those determinants in the analysis. This together will allow us to detect effects of age-related cardiovascular and metabolic phenotypes.

Deep phenotyping follow-up study

Recruitment and clinical examinations

A search of the Danish civil registration register (CPR register) in December 2019 showed that 6,004 (88.5%) of the Inter99 participants were alive and eligible for invitation (**Figure 1**).

All participants will be invited in the same order as examined at baseline to the clinical examination at The Center for Clinical Research and Prevention (CCRP) in Glostrup, Denmark. The first participant was examined on September 13, 2021. The data are collected in a highly standardized manner by trained health professionals. Data include repeated measures of the original health examinations (**Table 1)**, studies of sub-clinical signs of early cardiometabolic changes using innovative technologies, lifestyle questionnaires, extended sub-studies of diet, activity, and glucose (the InterDAG study) and a vitamin K supplementation intervention (InterVitaminK trial) as well as collection of biological samples for a biobank (**Figure 2)** as described below and in detail in **Supplemental Methods**.

- *Anthropometry and body composition*
  Height is measured without shoes to the nearest cm, weight without shoes and overcoat to the nearest kg, and body mass index (BMI) calculated (kg/m^2). Waist and hip circumferences are measured in cm using a non-stretchable tape and waist-to-hip ratio calculated. Segmental body composition is estimated from multi-frequency bioelectrical impedance analysis (InBody770, Biospace, Seoul, South Korea).

- *Arterial stiffness*
  Arterial stiffness is assessed by the gold standard method of assessing direct arterial stiffness based on carotid-femoral Pulse Wave Velocity (cfPWV) using the SphygmoCor XCEL instrument (AtCor Medical, Sydney, Australia) [25].

- *Biobank*
  Serum, plasma, and plasma urine will be collected from all participants. In a subgroup participating in the extended study on Diet, Activity and Glucose (InterDAG study) 24-hour urine samples and a fecal sample will be collected. This biobank allows for future analyses of selected biomarkers and multi-omics.

- *Blood biochemistry*
  Fasting blood samples (minimum of six hours) (**Table 1**) are collected and analyzed within three hours (**Supplemental Methods).**

- *Blood pressure*
  Blood pressure is measured thrice with an electronic blood pressure monitor (Microlife, Widnau, Switzerland) and fitting cuff after five minutes rest in sitting position.

- *Cardiac autonomic neuropathy*
  As a measure of cardiac autonomic neuropathy, simple bedside tests using resting heart rate variability (HRV) indices or response in heart rate to standing, slow breathing, or the Valsalva maneuver (cardiovascular autonomic reflex tests [CARTs]) is used with a Vagus device (Medicus Engineering, Aarhus, Denmark) [40,41].

- *Cardiac CT*
  All participants are offered a cardiac computerized tomography (CT) scan at the Department of Cardiology, Rigshospitalet in Copenhagen to determine coronary atherosclerosis, including coronary artery calcification score (CAC score), coronary stenosis, vascular extent and plaque type in addition to cardiac chamber size and left ventricular hypertrophy. The cardiac CT scan includes a non-contrast CT scan and a CT angiography performed using the 320-multidetector scanner (Aquilion One, Toshiba Medical Systems, Japan) [42,43]. In addition to cardiac risk assessment, the CT scan includes comprehensive imaging of lungs, vascular system, sarcopenia assessment, liver, spleen, abdominal fat, and spine.

- *Covariates*
  At baseline participants answered a questionnaire on sex, age, marital status, occupation, education, health (diagnoses of, e.g., cancer, diabetes, hypertension, high cholesterol, myocardial infarction, stroke, or coeliac disease) and lifestyle (physical activity, smoking, alcohol, and dietary habits) [29]. At follow-up participants answer additional questions on sarcopenia symptoms, sleep, sedentary behavior and physical activity [44–46].

Heart rhythm
Electrocardiography (ECG) is performed using 12 electrodes, recorded at 500Kz with a 10 second duration using the Cardiosoft system (GE Healthcare, Milwaukee, WI, USA). The Marquette 12SL algorithm (v. 21, GE Healthcare, Milwaukee, WI, USA) is used to obtain median beats as well as markers for P-wave/QRS wave onset and offset (QRS_{on},QRS_{off}) and T-wave offset (T_{off}) [47]. ECG diagnoses are made by the 12SL algorithm.

- *Liver stiffness and steatosis*

Noninvasive assessment of liver stiffness by transient elastography (TE) is performed after a minimum of 4-hrs fasting (FibroScan 502 Touch, Echosens, Baarn, Netherlands). The FIB-4 (Fibrosis-4) score will be calculated [48].

- *Muscle strength (hand grip and chair stand)*
  Muscular fitness is assessed using standardized protocols of muscle performance in the upper and lower extremity. Hand grip is measured using a Jamar® dynamometer (Sammons Preston Rolyan, Chicago, IL, USA) [49]. Lower body muscle performance is measured using the Sit-to-Stand test (STS) [50].

- *Oxygen saturation*
  Oxygen saturation is determined by direct measurement with the Nellcor™ portable SpO_2 pulse oximeter after blood sampling and 0 minutes rest in the supine position. Measurements is taken in the index finger of the opposite arm of blood sampling (Medtronic, Minneapolis, MN, USA).

- *Fundus characteristics*
  Ocular wide-field fundus photography and optical coherence tomography (OCT) are made in both eyes using the Optos Monaco device (Optos PLC, Dunfermline, UK) [51]. Images and scans are graded for retinopathy according to a modified version of the 'Proposed International Clinical DR severity scale' [52] applying a deep learning algorithm by convolutional neural networks [53].

- *Urine*
  A spot urine sample is collected and analyzed for sodium, potassium, albumin, and creatinine concentration.

Extended clinical studies

The study serves as a recruitment platform for the InterVitaminK Randomized Controlled Trial [25], as well as for the extended lifestyle and diurnal plasma glucose profiling InterDAG study as described below and in details in the **Supplemental Methods**.

InterVitaminK trial

All participants with detectable coronary arterial calcification (CAC≥10 Agatston units) assessed by the cardiac CT scan are invited to participate in the double-blinded placebo-controlled randomized intervention trial, the InterVitaminK trial (**Table 1, Figure 2**) as previously outlined [25].

InterDAG study (Diet, Activity, and Glucose)

Glucose levels are measured continuously for 10 days using continuous glucose monitoring (CGM) (Dexcom G6 PRO, Hudson, OH, USA). Simultaneously, comprehensive data on dietary intakes are collected, including urinary sodium and potassium, and physical activity simultaneous collection of a 7-day food record (MyFood24, www.myfood24.org), 10-day physical activity (24-hour Sens Motion® accelerometers, www.sens.dk, Copenhagen, Denmark). Participants are also invited for a 3-day repeated 24-hour urine collection and one single fecal collection for later analyses.

The simultaneous measurements of daily physical activity and diet will increase our understanding of the extent to which current lifestyle in older people influence, not only glucose regulation and variability, but also cardiometabolic health in general, including ectopic fat deposition, sarcopenia, and early vascular dysfunctions. The 24-hour urine collection will provide unique data on the sodium to potassium ratio, while the gut microbiota from fecal samples will provide insights into interaction between microbiota and metabolic diseases [54–56].

Register-based follow-up of the Inter99 cohort

The Inter99 cohort is linked to the nationwide Danish registries by the unique Danish CPR number. The registries cover hospital admissions, outpatient contacts, primary health care use, reimbursement of medicine, and a variety of social parameters (education, income, employment, ethnicity etc.). Information on date of T2D diagnosis is obtained from a newly established Danish Diabetes Register (DMreg) based on comprehensive data from the National Patient Register [57], the Medicines Products Register [58], the National Health Service Registry [59], the Danish Adult Diabetes Database [60], and the Eye Examination Database [61]. The algorithm calculating the date of diabetes diagnosis is described elsewhere [62]. Clinical and biochemical data to estimate trajectories following T2D diagnosis will be obtained from LABKA (Clinical Laboratory Information System Research Database) [63]. Information on date of various CVD and cerebrovascular disease diagnoses is based on the Danish National Patient Register [64], using International Classification of Diseases (ICD-10) codes. CVD is defined as atrial fibrillation, heart failure, hypertensive disease, ischemic heart disease. Cerebrovascular disease is defined as haemorrhagic stroke, ischemic stroke, and transient cerebral ischemia. Occurrence of macrovascular atherosclerotic disease will also be available from the registers.

Data analysis plan

As described in Table 1, this study will provide a wealth of data and future analyses strategies will depend on the outcome in focus. The statistical methods described below serves as an example of the methods most likely to be used, while alternative approaches will be applied when appropriate.

Statistics

For the register-based follow-up studies, Poisson regression and other time to event models (e.g., Cox proportional hazards models) will be applied to estimate incidence rates and hazard ratios with 95% confidence intervals, respectively, of clinical outcomes like T2D and CVD. Relevant covariates such as socioeconomic factors, adult BMI, and gene risk scores of cardiometabolic morbidity and obesity will be adjusted for in separate models. In other analysis, we will use multilevel longitudinal modelling to estimate clinical trajectories of markers of glycated hemoglobin, lipid levels, blood pressure, body mass index and kidney function as a function of various lifestyle-related and perinatal (e.g., birthweight) exposures.

For the 20-year cardiometabolic outcome follow-up study both binary (e.g., hypertension and retinopathy), categorial (e.g., sarcopenia and muscle strength) and continuous outcomes (e.g., CAC score, fibrosis score, polygenetic risk scores, and body composition) will be investigated. We will

use multiple logistic regression for binary outcomes, multinomial logistic regression for categorical outcomes and multiple linear regression for continuous outcomes. For each outcome, a series of models will be developed based on a priori knowledge about the causal framework around the association of interest. To assess the strength and direction of associations, we will report odds ratios and regression coefficients with corresponding 95% confidence intervals.

We will apply causal epidemiological techniques to identify and quantify the causal relationships of various lifestyle-related, genetic, and perinatal (e.g., birthweight) exposures with cardiometabolic outcomes, while predictive modeling will be applied to develop algorithms that can predict future disease risk. While classical statistical prediction modelling will be using risk factors identified from existing literature, machine learning techniques will be applied to develop prediction algorithms using a much wider spectrum of the available of data. As the clinical relevance has been an integral part of this study from the beginning, we aim to develop a series of interactive clinical tools that apply the developed prediction models for various cardiometabolic disease outcomes such as a T2D and CVD risk engine calculators. The combination of the wealth of cardiometabolic deep phenotyping data from the ongoing 20-year follow-up, polygenetic risk scores, lifestyle factors and perinatal factors such as objectively measured birth weight in a large sample of aging adults, provides a hitherto unparalleled potential for the development of real personalized risk prediction tools.

Ethics

Informed consent is obtained from all participants before clinical examinations and the study is conducted in accordance with the Declaration of Helsinki II and approved by the ethical committee of the Capital Region, Denmark (Inter99 Follow-up H-21033114) and (InterVitaminK trial H-20076231) and by the Danish Data Protection Agency (P-2020-1074). Clinicaltrials.gov registration: NCT05166447 and NCT05259046 respectively. Examinations are considered harmless, involve minimal inconvenience, and are performed by experienced healthcare professionals.

Perspectives

The Inter99 cohort has contributed substantially to our understanding of the multifactorial origin, natural history, as well as potential for early detection and prevention of T2D and its associated cardiometabolic co-morbidities. While it, from a modern epidemiologic perspective may appear relatively small, the Inter99 cohort is firmly established as an international competitive cardiometabolic epidemiological research resource due to its high data quality and data richness. As such, there is a strong foundation and a very high potential to perform a 20-year follow-up study of the now 50- to 80-year-old Inter99 participants.

So far, clinical reexaminations of the Inter99 cohort have been performed after 1, 3 and 5 years, and registry follow-up studies after 10 years. The planned 20-years follow-up study will include a combination of innovative cardiometabolic deep-phenotyping clinical examinations combined with comprehensive Danish national registry follow-up studies. While the Danish registries captures overt T2D and co-morbidity diagnoses, as well as use of medications, hospital admissions, selected

biochemical analyses, etc., a parallel and synergistic deep phenotyping study will allow us to determine several of the most import early disease manifestations present even prior to any official and often arbitrary cardiometabolic disease diagnosis criteria. Accordingly, the complimentary study approaches will enable us to quantify the extent to which the T2D associated co-morbidities coronary arteriosclerosis, cardiac autonomic neuropathy, coronary calcification, NAFLD, retinopathy, and diabetic kidney disease are present among elderly people without T2D and with a known normal glucose tolerance for two decades. The existing comprehensive genetic, birthweight, and lifestyle information, collected and analyzed over 20 years, provides unparalleled opportunities to determine how individual or groups of risk factors affect the natural history of overt and/or preclinical disease manifestations during the most relevant age window with the highest occurrence rates of T2D and associated cardiometabolic diseases. The complementary data most importantly increases our signal to noise ratio, and thus statistical power, to detect the relative contribution of a variety of distinct risk factors. For instance, having near complete GWAS data allows adjustment for putative genetic confounders influencing associations between birthweight and overt or preclinical disease. All pieces of information with high importance to understand the heterogeneity, and thus for driving, innovating, and implementing precision medicine, of T2D and co-morbidities.

The major etiological factors underlying risk of T2D, and its co-morbidities include genetics on one side, and pre- and postnatal environmental exposures on the other (**Box 1**). As for risk factors in pregnancy, the remarkably most accurate marker predicting cardiometabolic disease is weight at the time of birth [65,66]. While there has been much focus on identifying more specific exposures underlying the association between LBW and disease risk, no single factor influencing fetal growth during pregnancy has yet been identified to explain the association. In contrast, multiple epidemiological and animal studies have documented that virtually all factors in pregnancy that negatively influence fetal growth and birthweight including maternal smoking, diet, and energy intake, reduced placental blood flow etc. are associated with increased risk of cardiometabolic diseases in the offspring [67]. Accordingly, rather than representing only a risk marker, LBW may represent a mediator of the totality of adverse events and lifestyle factors in pregnancy that influence later risk of cardiometabolic diseases in the offspring, justifying birthweight as the so far unparalleled cardiometabolic risk marker of prenatal disease exposures.

Based on the Inter99 baseline health examinations, we previously confirmed LBW to be associated with T2D prevalence at a mean age of only 46 years [35]. As the first 20-year follow-up initiative, we studied the association between birthweight and T2D incidence rates [68]. Using the Danish registries, birth records of 4,590 Inter99 participants were linked with age at T2D diagnosis, as well as relevant covariates. We identified 492 new T2D cases since 1999, and subsequently documented that T2D incidence rate decreased with increasing birthweight in a surprisingly linear manner [68]. Interestingly, our study clearly supported the notion that the other major etiological factors of genetics and obesity appeared to operate as independent and most likely additive risk factors on top of that of lower birthweight [68].

Further comprehensive registry analyses of the full range of T2D vascular complications and co-morbidities will provide unparalleled insights into previously unrecognized differential T2D and co-

morbidity sub-phenotypes and their underlying etiologies. Here, we for example can determine the extent to which T2D patients with the lowest birthweights may be characterized by a more severe clinical presentation as recently suggested [13]. To improve our understanding of T2D and its sub-phenotypes, similar analytical strategies will be applied for the various early disease markers and manifestations determined in the deep phenotyping clinical follow-up study.

The extended InterDAG subgroup study is aiming to better understand (and adjust for) the impact of diet, whole body sodium and potassium balance, as well as physical activity, on diurnal glucose levels and fluctuations across a wide range of the glucose tolerance spectra. Blood samples available from the baseline health examinations, along with samples from the reexaminations, will be available for extended micronutrient and multi-omics analyses including whole genome sequencing, metabolomics, lipidomics, transcriptomics, epigenomics, proteomics, and metagenomics. Our vision includes extensive application of AI based analyses to integrate the clinical, biochemical, and genetic data over time, across and beyond current clinical diagnostic cardiometabolic disease criteria.

The Inter99 20-year follow-up study furthermore provides a unique opportunity to study age-related outcomes, such as sarcopenia and physical function. It is well established that lifestyle (diet, physical activity, smoking and BMI) influences the risk of chronic disease, thus Inter99 20-year data will allow for the study of long-term impact of lifestyle in early adulthood on subsequent age-related disease manifestations. These data will for instance allow us to study the trends in dietary habits and physical activity patterns and their impact on muscle strength and function in middle- and old age in people with and without T2D or CVD. As such, our Inter99 follow-up study will provide important insights into the mechanisms underlying age-related processes in both healthy and diseased individuals.

In conclusion, the current combined epidemiological registry and deep phenotyping 20-year clinical follow-up study provides an example of the value of reexamining an existing and already extensively characterized T2D and cardiometabolic cohort, with the overall aim to better understand etiologically distinct disease trajectories and sub-phenotypes. This will facilitate development of better and more efficacious precision medicine prediction, clinical care, as well as overall treatment approaches in T2D and associated diseases. The cohort data will via a scientific steering group be available for international collaborations.

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Author contributions

KB and CB drafted the first version of the manuscript. AV and AL initiated the study. KB, CB, MA, FBK, CFBN, BL, RW, CS, KN, NRJ, CSU, MK, NG, JK, LML, LK, ALM, KFK, RL, TH, AL, AV designed and managed the study. All authors critically reviewed the manuscript, read, and approved the final version of the manuscript.

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Competing interest

None declared.

Patient and public involvement

Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

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Table 1. Summary of data collected at the Inter99 baseline and the 20-year follow-up study (including extended sub-studies).

Data	Variables	Baseline	20-year
Questionnaire-based information			
Demographics	Sex, age, family, marital status, education, employment status, household income	X	X
Diseases	Chronic diseases, contact to health care system, symptoms	X	X
Health	Self-rated health, stress, sleep	X	X
Lifestyle	Physical activity, smoking, alcohol, diet, network	X	X
Deep-phenotyping health assessment			
Anthropometry	Height, weight, waist and hip circumference	X	X
Bioelectrical impedance	Fat and lean (muscle) body mass	X	X
Blood pressure	Systolic and diastolic blood pressure, resting heart rate	X	X
Cardiac autonomic neuropathy	Resting heart rate variability and cardiovascular autonomic reflex tests		X
Cardiac CT	Coronary atherosclerosis, cardiac chamber size, LV hypertrophy		X
Continuous glucose monitoring	7-day 24-hour glucose levels		X
Dynamometer and sit-to-stand test	Muscle strength		X
Electrocardiography	Heart rhythm and pathologies		X
Optos scanning	Ocular fundus characteristics, retinopathy	X	X
Oxymeter	Oxygen saturation		X
Pulse wave velocity	Arterial stiffness		X
Spirometry	Lung function	X	(S)
Transient elastography	Liver stiffness and steatosis		X
Laboratory assessments			
Blood biochemistry	Leukocytes and differential count, electrolytes (sodium), glucose, HbA1c, lipids (total cholesterol, HDL, LDL, VLDL, triglycerides), kidney function (creatinine, eGFR), Vitamin K status (dephosphorylated-uncarboxylated matrix-gla Protein), liver function (ALAT, ASAT)	X	X
Urine biochemistry	Albumin, creatinine, sodium, potassium	X	X
Biobanking (-80°C)			
Blood	Fasting blood samples (whole-blood, serum, and plasma)	X	X
Urine	Spot urine 24-hour urine collection	X X(S)	X X(S)
Feces	Fecal samples		X(S)

(S) subgroup of participant

Figure 1.

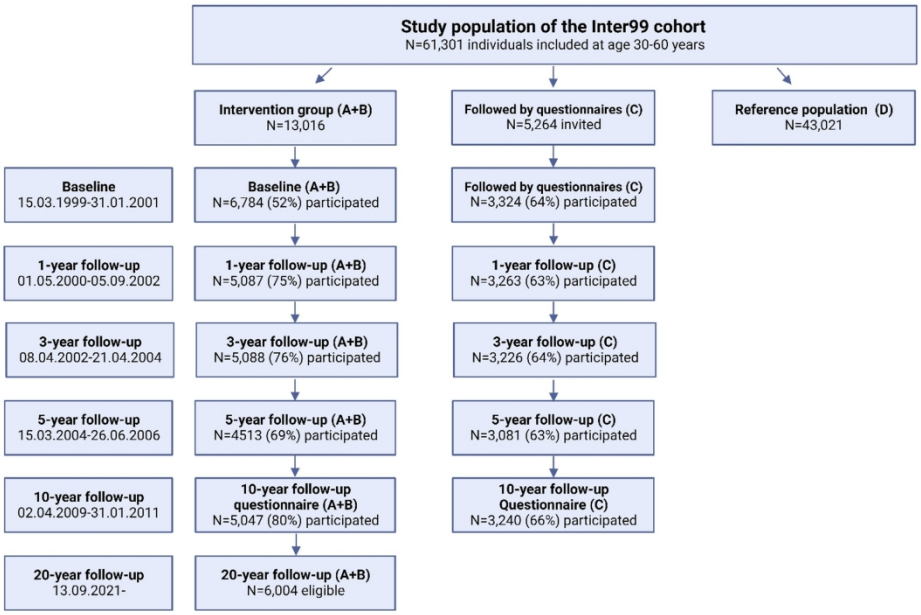

Figure 1. Flow chart of particicipation in the Inter99 study 1999–2023. From a study population of N=61,301 individuals, N=13,016 and N=5,264 were randomized into two intervention groups (A+B) and a group followed by questionnaires (C). The A and B intervention groups participated in health examination, questionnaires, and lifestyle interventions, while group C was followed only by questionnaires. A reference population (D) of N=43,021 individuals were followed in registries and not contacted. Participants from the intervention groups (A+B), who participated in the baseline study and were eligible in December 2019 are invited for the Inter99 20-year follow-up; N=6,004.

188x128mm (300 x 300 DPI)

Figure 2.

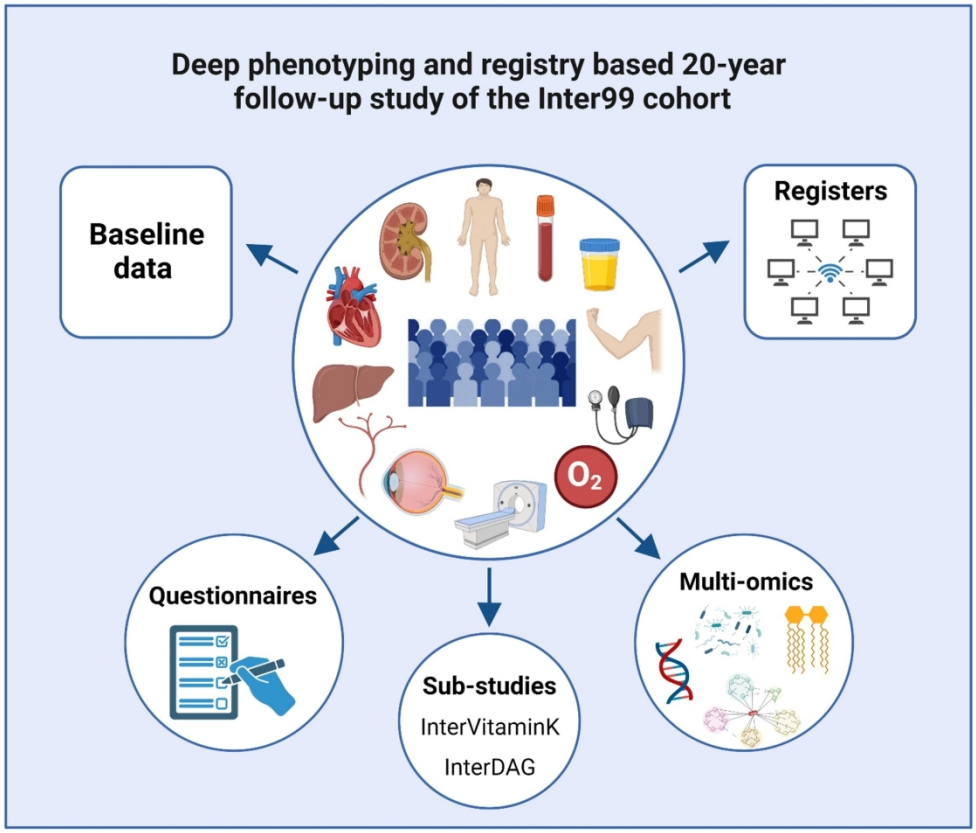

Figure 2. Overview of the 20-year deep-phenotyping follow-up clinical examinations, the possibilities of coupling to baseline and register data as well as for future and extended analyses.

183x167mm (300 x 300 DPI)

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

The total of cholesterol, triglyceride and high-density lipoprotein cholesterol (HDL) will be sampled in heparin tubes and centrifuged, whereby content is determined in plasma with colorimetric slide test (Vitros 4600/5600, Ortho Clinical Diagnostics, Raritan, USA). Very-low density lipoprotein cholesterol (VLDL) and low-density lipoprotein cholesterol (LDL) is calculated from VLDL = 0.45* triglyceride; LDL = Total Cholesterol-HDL-VLDL. Blood samples for glucose measurements will be taken in citrate buffer-fluoride mixture (FC-Mixture) tubes and measured by HPLC. Aldosterone and renin will be sampled in EDTA tubes, centrifuged and plasma stored at -80℃, before analysis by iSYS equipment from IDS PLC, Tyne and Wear, UK.

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, Canon 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].

Heart rate variability

No human overreading will take place. The RR interval, QRS duration, and QT interval will be obtained. The QT interval will be corrected for heart rate using the method of Fridericia (QTcF). The ECGs will be converted to VCGs by two different transformation matrices: the Kors and the Inverse Dower matrices [2,3]. A QRS-Ta estimate will be obtained without VCG transformation by the method of Rautaharju [4].

Muscle Strength (hand grip and chair stand)

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.
- 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
    - Cardiac autonomic neuropathy
    - Coronary artery calcification
    - Fundus characteristics
    - Heart rhythm
    - Liver stiffness and steatosis
    - Muscle strength
    - Oxygen saturation

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5-6
Objectives	3	State specific objectives, including any prespecified hypotheses	5-6
Methods			
Study design	4	Present key elements of study design early in the paper	5-7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6-7
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6-7
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	-
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7-9
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	7-9
Bias	9	Describe any efforts to address potential sources of bias	5
Study size	10	Explain how the study size was arrived at	7
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	10-11
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	10-11
		(*b*) Describe any methods used to examine subgroups and interactions	10-11
		(*c*) Explain how missing data were addressed	10
		(*d*) If applicable, explain how loss to follow-up was addressed	10
		(*e*) Describe any sensitivity analyses	-
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	6-7
		(b) Give reasons for non-participation at each stage	n/a
		(c) Consider use of a flow diagram	n/a
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	-
		(b) Indicate number of participants with missing data for each variable of interest	-
		(c) Summarise follow-up time (eg, average and total amount)	7
Outcome data	15*	Report numbers of outcome events or summary measures over time	-
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	-
		(*b*) Report category boundaries when continuous variables were categorized	-
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	-
Discussion			
Key results	18	Summarise key results with reference to study objectives	-
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	-
Generalisability	21	Discuss the generalisability (external validity) of the study results	-
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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

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

Kirsten Bjørnsbo[1*], Charlotte Brøns[2*], Mette Aadahl[1], Freja Bach Kampmann[1], Camilla Friis Bryde Nielsen[1], Bjørn Lundbergh[1], Rasmus Wibaek[2], Line Kårhus[1], Anja Lykke Madsen[1], Christian Stevns Hansen[2], Kirsten Nørgaard[2,3], Niklas Rye Jørgensen[4], Charlotte Suetta[5], Michael Kjær[5], Niels Grarup[6], Jørgen K. Kanters[4,7], Michael Larsen[3,8], Lars Køber[9], Klaus F. Kofoed[3,9], Ruth J.F. Loos[6], Torben Hansen[6], Allan Linneberg[1,3**], Allan Vaag[2,10,11**]

*Contributed equally to this work

**Shared last authorship

[1] Center for Clinical Research and Prevention, Copenhagen University Hospital – Bispebjerg and Frederiksberg, Copenhagen, Denmark
[2] Steno Diabetes Center Copenhagen, Herlev, Denmark
[3] Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
[4] Department of Clinical Biochemistry, Copenhagen University Hospital – Rigshospitalet, Copenhagen, Denmark
[5] Institute of Sports Medicine, Dept of Orthopedic Surgery and Department of Geriatrics and Palliative Medicine, Copenhagen University Hospital – Bispebjerg and Frederiksberg, Copenhagen, Denmark
[6] Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[7] Center of Physiological Research, University of California San Francisco, San Francisco, CA, USA
[8] Department of Ophthalmology, Copenhagen University Hospital – Rigshospitalet, Glostrup, Denmark
[9] Department of Cardiology and Radiology, Copenhagen University Hospital – Rigshospitalet, Copenhagen, Denmark
[10] Lund University Diabetes Centre, Malmö, Sweden
[11]Dept. of Endocrinology, Skåne University Hospital, Malmö, Sweden.

Corresponding authors: Kirsten Bjørnsbo kirsten.bjoernsbo@regionh.dk and Charlotte Brøns charlotte.broens.01@regionh.dk

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Abstract

Introduction The population-based Inter99 cohort has contributed extensively to our understanding of effects of a systematic screening and lifestyle intervention, as well as the multifactorial etiology of type 2 diabetes (T2D) and cardiovascular disease (CVD). To understand causes, trajectories, and patterns of early and overt cardiometabolic disease manifestations, we will perform a combined clinical deep phenotyping and registry follow-up study of the now 50-80 years old Inter99 participants.

Methods and analysis The Inter99 cohort comprise individuals aged 30-60 years, who lived in a representative geographical area of greater Copenhagen, Denmark, in 1999. Age- and sex-stratified random subgroups were invited to participate in either a lifestyle intervention (N=13,016) or paper surveys (N=5,264), while the rest served as a reference population (N=43,021). Of the 13,016 individuals assigned to the lifestyle intervention group, 6,784 (52%) accepted participation in a baseline health examination in 1999, including screening for cardiovascular risk factors and prediabetic conditions. In total, 6,004 eligible participants who participated in the baseline examination, will be invited to participate in the deep phenotyping 20-year follow-up clinical examination including measurements of anthropometry, blood pressure, arterial stiffness, cardiometabolic biomarkers, coronary artery calcification, heart rate variability, heart rhythm, liver stiffness, fundus characteristics, muscle strength and mass, as well as health and lifestyle questionnaires. In a subsample, 10-day monitoring of diet, physical activity and continuous glucose measurements will be performed. Fasting blood, urine, and fecal samples to be stored in a biobank. The established database will form the basis of multiple analyses. A main purpose is to investigate whether low birthweight independent of genetics, lifestyle and glucose tolerance predicts later common T2D cardiometabolic co-morbidities.

Ethics and dissemination The study was approved by the Medical Ethics Committee, Capital Region, Denmark (H-20076231) and by the Danish Data Protection Agency through the Capital Region of Denmark's registration system (P-2020-1074). Informed consent will be obtained before examinations. Findings will be disseminated in peer-reviewed journals, at conferences, and via presentations to stakeholders, including patients and public health policymakers.

Study registration: ClinicalTrials.gov, NCT05166447.

Strengths and limitations of this study

- The longitudinal design will enable us to follow the course of both early and overt cardiometabolic disease manifestations during the period of life with highest incidence rates.
- We will be able to quantify the extent to which the type 2 diabetes (T2D)-associated co-morbidities coronary arteriosclerosis, cardiac autonomic neuropathy, non-alcoholic fatty liver disease, retinopathy, and diabetic kidney disease are present among elderly people without T2D, and with known normal glucose tolerance for two decades.
- The availability of genome-wide genetic variation data, birthweight, as well as adiposity trajectories, dietary data, biomarkers of micronutrient status, and physical activity information, will provide insights into how these predisposing factors influence distinct organ morbidities and disease manifestations.
- Collection of biospecimens for micronutrient and multi-omics purposes such as genomics, transcriptomics, proteomics, metabolomics, lipidomics, and epigenomics will allow additional layers of deep phenotype analyses, including studying disease-associated genetic variants and their phenotypes.
- The fact that all individuals participated in a screening for cardiovascular disease risk and, if at risk, invited for a personalized lifestyle intervention from 1999 and for up to five years thereafter, may limit the generalizability of our findings.

INTRODUCTION

Type 2 diabetes (T2D), cardiovascular disease (CVD) and their co-morbidities are leading causes of premature mortality and morbidity, affecting nearly one billion individuals worldwide [1,2]. T2D is arbitrarily defined by elevations of plasma glucose levels, and the current T2D diagnostic criteria does not capture the diversity of T2D sub-phenotypes characterized by differential manifestations of micro - and macrovascular complications, as well as other common comorbidities [3,4].

The overlap and heterogeneity of age-related T2D, CVD and associated comorbidities are likely rooted in the relative or predominant contributions from the triad of genetic susceptibility versus pre- and postnatal non-genetic etiologies (**Box 1**).

Box 1. The complex multifactorial pre- and postnatal etiology of cardiometabolic diseases

Constitutional primary predisposing factors

- Genetic susceptibility
- Intrauterine environment (low or high birth weight, prematurity)

Acquired postnatal secondary precipitating factors

- Sedentary lifestyle / inactivity
- Unhealthy diet / micronutrient deficiencies
- Obesity
- Smoking
- Medication
- Comorbidities
- Aging

As for genetics, the known 568 T2D susceptibility loci are estimated to explain 18% of the putative genetic contribution to T2D [5,6]. Early life developmental programming, low birth weight (LBW), as well as salt-sensitive hypertension, non-alcoholic fatty acid disease (NAFLD), dyslipidemia, and neurocognitive dysfunctions, are well-established risk factors of T2D and CVD [7–12]. Recent data even suggest that LBW, in a non-genetic manner, is associated with a more severe clinical T2D presentation and course, including earlier onset and more comorbidities at the time of diagnosis [13]. Accordingly, there is an increasing need to understand whether differential combinations of T2D etiologies influence not only T2D and CVD risk *per se*, but also the timing and patterns of clinical presentation including both early and late disease manifestations, as well as co-morbidities. As an example of unprioritized co-morbidities, T2D patients have a two-three-fold increased risk of sarcopenia [14,15]. Sarcopenia describes the age-related loss of muscle mass and strength that leads to impaired function including increased risk of falls and an overall decreased quality of life. Sarcopenia is accelerated by physical inactivity, low protein intake, and general health status and disease, and has also been associated with LBW [16].

Excess dietary sodium (Na^+) may account for three million deaths annually [17], and reducing salt intake is among the most cost-effective CVD prevention strategies [18]. Low dietary potassium (K^+) intake is also gaining attention as a CVD risk factor, and the urinary Na^+/K^+-ratio may therefore represent a superior cardiovascular risk measure [19–23]. As for macronutrient intake, high dietary

sugar and saturated fat contents is strongly associated with T2D and CVD risk. While the Mediterranean diet may prevent CVD [24], there is nevertheless substantial gaps in our current knowledge of what defines a healthy diet with respect to not only macro but also micro nutritional composition(s) including vitamins. For instance, beyond effects on blood clotting factors, vitamin K may be important for cardiometabolic as well as bone health [25].

The Inter99 cohort provides a unique research platform to delineate the differential and overlapping roles of genetics versus the fetal environmental, as well as various postnatal lifestyle factors, for the development of early and overt cardiometabolic disease manifestations and associated co-morbidities [26–28]. We aim to perform a combined deep phenotyping and registry-based follow-up study of the Inter99 cohort, 20 years after the baseline health examinations, when participants were on average 46 years of age. While overt disease diagnoses will be captured by Danish national registries, our deep phenotyping clinical examinations allow detection of a wider range of early disease manifestations, such as vascular stiffness, liver fibrosis, retinopathy, diabetic kidney disease, and prediabetes, and the extent to which these may be present prior to the participants complying with official cardiometabolic disease diagnoses.

METHODS AND ANALYSIS

Study setting and previous findings

The Inter99 study was initiated in March 1999 as a population-based multi-factorial intervention study, originally designed to prevent ischemic heart disease (IHD) [29]. It comprised all individuals born in 1939-40, 1944-45, 1949-50, 1954-55, 1959-60, 1964-65, and 1969-70 (30, 35, 40, 45, 50, 55, and 60 years of age) living in 11 municipalities in Greater Copenhagen (N=61,301). The population was randomized with different age and sex ratios to two lifestyle intervention groups (A+B; N=13,016) or a group followed by questionnaires (C; N=5,264), the remaining individuals were considered as a reference population and not contacted (D; N=43,021) (**Figure 1**). Participants received individualized lifestyle counseling based on lifestyle and CVD risk score [30]. In total, 6,784 participants from the intervention groups participated in the baseline health examination (52% acceptance rate) [29]. The 5-year clinical follow-up examination, including glucose tolerance status, had a participation rate of 69% [31]. The 10-year follow-up was based on registry data and a follow-up questionnaire was sent to all eligible participants with completed baseline health examination (A+B) and to all in the paper survey group (C) (**Figure 1**) [32].

The 5-year follow-up examination showed a progression rate to overt T2D of 2.1 per 100 person-years [33], strongly supported by a substantially higher T2D prevalence after 20 years of follow-up with 6.5% of men and 3.8% in women having been diagnosed with T2D. Indeed, this will also be the case for all other T2D-associated vascular and cardiometabolic co-morbidities, underscoring the relevance of performing a combined registry-based and cardiometabolic deep phenotyping study among Inter99 participants, who are now 50-80 years old.

Original midwife records were collected from 4,744 participants in the intervention groups [34,35], and despite the relatively low average age of 46 years in 1999, we confirmed a strong inverse relationship between birthweight and risk of T2D in this Danish population [35]. The Inter99 cohort has been extensively genotyped contributing to the identification of more than 568 T2D susceptibility loci [5], and to the interactions between birthweight and genetic risk of T2D [27,28,36]. The

prevalence of T2D associated retinopathy in the Inter99 cohort was studied in a subgroup of 970 participants. Interestingly, retinopathy was present in 7.5% of the 490 subjects with completely normal glucose tolerance, supporting the notion that factors other than elevated glucose contributes to the risk of retinopathy of a type that cannot be distinguished from milder degrees of diabetic retinopathy [37].

The 10-year follow-up study concluded that a community-based, individually tailored intervention program with screening for risk of ischemic heart disease and repeated lifestyle intervention over 5 years, had no effect on ischemic heart disease, stroke, or mortality at the population level as assessed after 10 years [38]. This observation was later reproduced in other studies and confirmed in a WHO report [39].

The totality of data already available in the Inter99 cohort range from information on birth size (weight and length) and prematurity, glucose tolerance at baseline and at 5-year follow-up, lifestyle intervention and general health information including comprehensive dietary data, numerous biomarkers, and genetic data (**Table 1, Figure 2**). The 20-year follow-up study includes physical deep phenotyping clinical examinations tailored to capture and expand our growing understanding of T2D and subgroups, as well as early and late metabolic and vascular manifestations, co-morbidities, and complications. Morbidity and mortality data is obtained from our extensive Danish registers. The existing detailed clinical and lifestyle information over 20 years will allow us to correct for those determinants in the analysis. This together will allow us to detect effects of age-related cardiovascular and metabolic phenotypes.

Deep phenotyping follow-up study

Recruitment and clinical examinations

A search of the Danish civil registration register (CPR register) in December 2019 showed that 6,004 (88.5%) of the Inter99 participants, who had participated in the baseline examination, were alive and had not emigrated; and thus, eligible for inclusion (**Figure 1**). There were no other eligibility criteria for study participation.

All participants will be invited in the same order as examined at baseline to the clinical examination at The Center for Clinical Research and Prevention (CCRP) in Glostrup, Denmark. The first participant was examined on September 13, 2021. The data are collected in a highly standardized manner by trained health professionals. Data include repeated measures of the original health examinations (**Table 1)**, studies of sub-clinical signs of early cardiometabolic changes using innovative technologies, lifestyle questionnaires, extended sub-studies of diet, activity, and glucose (the InterDAG study) and a vitamin K supplementation intervention (InterVitaminK trial) as well as collection of biological samples for a biobank (**Figure 2)** as described below and in detail in **Supplemental Methods**.

- *Anthropometry and body composition*

  Height is measured without shoes to the nearest cm, weight without shoes and overcoat to the nearest kg, and body mass index (BMI) calculated (kg/m^2). Waist and hip circumferences are measured in cm using a non-stretchable tape and waist-to-hip ratio calculated. Segmental body

composition is estimated from multi-frequency bioelectrical impedance analysis (InBody770, Biospace, Seoul, South Korea).

- *Arterial stiffness*

Arterial stiffness is assessed by the gold standard method of assessing direct arterial stiffness based on carotid-femoral Pulse Wave Velocity (cfPWV) using the SphygmoCor XCEL instrument (AtCor Medical, Sydney, Australia) [25].

- *Biobank*

Serum, plasma, and plasma urine will be collected from all participants. In a subgroup participating in the extended study on Diet, Activity and Glucose (InterDAG study) 24-hour urine samples and a fecal sample will be collected. This biobank allows for future analyses of selected biomarkers and multi-omics.

- *Blood biochemistry*

Fasting blood samples (minimum of six hours) (**Table 1**) are collected and analyzed within three hours (**Supplemental Methods).**

- *Blood pressure*

Blood pressure is measured thrice with an electronic blood pressure monitor (Microlife, Widnau, Switzerland) and fitting cuff after five minutes rest in sitting position.

- *Cardiac autonomic neuropathy*

As a measure of cardiac autonomic neuropathy, simple bedside tests using resting heart rate variability (HRV) indices or response in heart rate to standing, slow breathing, or the Valsalva maneuver (cardiovascular autonomic reflex tests [CARTs]) is used with a Vagus device (Medicus Engineering, Aarhus, Denmark) [40,41].

- *Cardiac CT*

All participants are offered a cardiac computerized tomography (CT) scan at the Department of Cardiology, Rigshospitalet in Copenhagen to determine coronary atherosclerosis, including coronary artery calcification score (CAC score), coronary stenosis, vascular extent and plaque type in addition to cardiac chamber size and left ventricular hypertrophy. The cardiac CT scan includes a non-contrast CT scan and a CT angiography performed using the 320-multidetector scanner (Aquilion One, Toshiba Medical Systems, Japan) [42,43]. In addition to cardiac risk assessment, the CT scan includes comprehensive imaging of lungs, vascular system, sarcopenia assessment, liver, spleen, abdominal fat, and spine.

- *Covariates*

At baseline participants answered a questionnaire on sex, age, marital status, occupation, education, health (diagnoses of, e.g., cancer, diabetes, hypertension, high cholesterol, myocardial infarction, stroke, or coeliac disease) and lifestyle (physical activity, smoking, alcohol, and dietary

habits) [29]. At follow-up participants answer additional questions on sarcopenia symptoms, sleep, sedentary behavior, and physical activity [44–46].

- *Electrocardiography (ECG)*
  Ten second 12-lead ECG is digitally recorded at 500 hz for 10 seconds and analyzed using the Marquette 12SL algorithm (v. 21, GE Healthcare, Milwaukee, WI, USA) [47].

- *Liver stiffness and steatosis*
  Noninvasive assessment of liver stiffness by transient elastography (TE) is performed after a minimum of 4-hrs fasting (FibroScan 502 Touch, Echosens, Baarn, Netherlands). The FIB-4 (Fibrosis-4) score will be calculated [48].

- *Muscle strength (hand grip and chair stand)*
  Muscular fitness is assessed using standardized protocols of muscle performance in the upper and lower extremity. Hand grip is measured using a Jamar® dynamometer (Sammons Preston Rolyan, Chicago, IL, USA) [49]. Lower body muscle performance is measured using the Sit-to-Stand test (STS) [50].

- *Oxygen saturation*
  Oxygen saturation is determined by direct measurement with the Nellcor™ portable SpO_2 pulse oximeter after blood sampling and 0 minutes rest in the supine position. Measurements is taken in the index finger of the opposite arm of blood sampling (Medtronic, Minneapolis, MN, USA).

- *Fundus characteristics*
  Ocular wide-field fundus photography and optical coherence tomography (OCT) are made in both eyes using the Optos Monaco device (Optos PLC, Dunfermline, UK) [51]. Images and scans are graded for retinopathy according to a modified version of the 'Proposed International Clinical DR severity scale' [52] applying a deep learning algorithm by convolutional neural networks [53].

- *Urine*
  A spot urine sample is collected and analyzed for sodium, potassium, albumin, and creatinine concentration.

Extended clinical studies

The study serves as a recruitment platform for the InterVitaminK Randomized Controlled Trial [25], as well as for the extended lifestyle and diurnal plasma glucose profiling InterDAG study as described below and in details in the **Supplemental Methods**.

InterVitaminK trial

In total, 450 men and women who participated in the Inter99 20-year follow-up study with detectable CAC (CAC≥10 Agatston units) assessed by the cardiac CT scan will be recruited to participate in the double-blinded placebo-controlled randomized intervention trial, the InterVitaminK trial,

investigating the effect of Vitamin K supplementation on progression of CAC (**Table 1, Figure 2**) as described in more detail elsewhere [25].

InterDAG study (Diet, Activity, and Glucose)

The InterDAG study will recruit 1000 consecutive participants from the Inter99 follow-up study with no exclusion criteria. Glucose levels are measured continuously for 10 days using continuous glucose monitoring (CGM) (Dexcom G6 PRO, Hudson, OH, USA). Simultaneously, comprehensive data on dietary intakes are collected, including urinary sodium and potassium, and physical activity simultaneous collection of a 7-day food record (MyFood24, www.myfood24.org), 10-day physical activity (24-hour Sens Motion® accelerometers, www.sens.dk, Copenhagen, Denmark). Participants are also invited for a 3-day repeated 24-hour urine collection and one single fecal collection for later analyses. The simultaneous measurements of daily physical activity and diet will increase our understanding of the extent to which current lifestyle in older people influence, not only glucose regulation and variability, but also cardiometabolic health in general, including ectopic fat deposition, sarcopenia, and early vascular dysfunctions. The 24-hour urine collection will provide unique data on the sodium to potassium ratio, while the gut microbiota from fecal samples will provide insights into interaction between microbiota and metabolic diseases [54–56].

Expected timeline

The cardiometabolic deep-phenotyping study including cardiac CT scans and 7-day continuous monitoring of diet, activity, and glucose (the InterDAG) in a sub-group will be completed in April 2024. Data cleaning, validation and organization of the database is expected to be completed by end 2024.

Register-based follow-up of the Inter99 cohort

The Inter99 cohort is linked to the nationwide Danish registries by the unique Danish CPR number. The registries cover hospital admissions, outpatient contacts, primary health care use, reimbursement of medicine, and a variety of social parameters (education, income, employment, ethnicity etc.). Information on date of T2D diagnosis is obtained from a newly established Danish Diabetes Register (DMreg) based on comprehensive data from the National Patient Register [57], the Medicines Products Register [58], the National Health Service Registry [59], the Danish Adult Diabetes Database [60], and the Eye Examination Database [61]. The algorithm calculating the date of diabetes diagnosis is described elsewhere [62]. Clinical and biochemical data to estimate trajectories following T2D diagnosis will be obtained from LABKA (Clinical Laboratory Information System Research Database) [63]. Information on date of various CVD and cerebrovascular disease diagnoses is based on the Danish National Patient Register [64], using International Classification of Diseases (ICD-10) codes. CVD is defined as atrial fibrillation, heart failure, hypertensive disease, ischemic heart disease. Cerebrovascular disease is defined as haemorrhagic stroke, ischemic stroke, and transient cerebral ischemia. Occurrence of macrovascular atherosclerotic disease will also be available from the registers.

Data analysis plan

As described in Table 1, this study will provide a wealth of data and future analyses strategies will depend on the research question and outcome in focus. The statistical methods described below serves as an example of the methods most likely to be used, while alternative approaches will be applied when appropriate.

As the established Inter99 20-year follow-up database will form the basis for testing several research questions, the analyses should be considered explorative in nature. However, a main hypothesis of the Inter99 20-year follow-up was to investigate whether low birthweight independent of genetics, lifestyle and glucose tolerance over 20 years is related to common T2D cardiometabolic co-morbidities. For the register-based follow-up studies, Poisson regression and other time to event models (e.g., Cox proportional hazards models) will be applied to estimate incidence rates and hazard ratios with 95% confidence intervals, respectively, of clinical outcomes like T2D and CVD. Relevant covariates such as socioeconomic factors, adult BMI, and gene risk scores of cardiometabolic morbidity and obesity will be adjusted for in separate models. In other analysis, we will use multilevel longitudinal modelling to estimate clinical trajectories of markers of glycated hemoglobin, lipid levels, blood pressure, body mass index and kidney function as a function of various lifestyle-related and perinatal (e.g., birthweight) exposures.

Both binary (e.g., hypertension and retinopathy), categorial (e.g., sarcopenia and muscle strength) and continuous outcomes (e.g., CAC score, fibrosis score, polygenetic risk scores, and body composition) will be employed. We will use multiple logistic regression for binary outcomes, multinomial logistic regression for categorical outcomes and multiple linear regression for continuous outcomes. For each outcome, a series of models will be developed based on a priori knowledge about the causal framework around the association of interest. To assess the strength and direction of associations, we will report odds ratios and regression coefficients with corresponding 95% confidence intervals.

We will apply causal epidemiological techniques to identify and quantify the causal relationships of various lifestyle-related, genetic, and perinatal (e.g., birthweight) exposures with cardiometabolic outcomes, while predictive modeling will be applied to develop algorithms that can predict future disease risk. The model and variable selection will depend on the research question. One approach is the concept of causal models and causal directed acyclic graphs. For some research questions it is also possible to use genetic risk scores as unbiased instruments of exposures. When optimal prediction of disease is the main purpose, models will be compared by using C-statistics and other related approaches.

As the clinical relevance has been an integral part of this study from the beginning, we aim to develop a series of interactive clinical tools that apply the developed prediction models for various cardiometabolic disease outcomes such as a T2D and CVD risk engine calculators. The combination of the wealth of cardiometabolic deep phenotyping data from the ongoing 20-year follow-up, polygenetic risk scores, lifestyle factors and perinatal factors such as objectively measured birth weight in a large sample of aging adults, provides a hitherto unparalleled potential for the development of real personalized risk prediction tools.

Patient and public involvement

None.

ETHICS AND DISSEMINATION

The study is conducted in accordance with the Declaration of Helsinki II and is approved by the ethical committee of the Capital Region, Denmark (Inter99 follow-up, H-21033114; InterVitaminK trial, H-20076231) and by the Danish Data Protection Agency (P-2020-1074). Informed consent is obtained from all participants before clinical examinations. Clinicaltrials.gov registration: NCT05166447 and NCT05259046, respectively. Examinations are considered harmless, involve minimal inconvenience, and are performed by experienced healthcare professionals. Performing CT-scans may result in incidental findings that need further examination and potentially treatment. As for other screening procedures this may cause both benefit (early detection and treatment) and harm (over-treatment) to the participants. The radiation dose associated with the CT-scan is relatively low and considered of minimal risk.

Findings will be disseminated in peer-reviewed journals, at national and international conferences, and via presentations to all interested stakeholders including patients and public health policymakers. Data may be made available for international collaborations upon request.

DISCUSSION

The Inter99 cohort has contributed substantially to our understanding of the multifactorial origin, natural history, as well as potential for early detection and prevention of T2D and its associated cardiometabolic co-morbidities. While it, from a modern epidemiologic perspective may appear relatively small, the Inter99 cohort is firmly established as an international competitive cardiometabolic epidemiological research resource due to its high data quality and data richness. As such, there is a strong foundation and a very high potential to perform a 20-year follow-up study of the now 50- to 80-year-old Inter99 participants.

So far, clinical reexaminations of the Inter99 cohort have been performed after 1, 3 and 5 years, and registry follow-up studies after 10 years. The planned 20-years follow-up study will include a combination of innovative cardiometabolic deep-phenotyping clinical examinations combined with comprehensive Danish national registry follow-up studies. While the Danish registries captures overt T2D and co-morbidity diagnoses, as well as use of medications, hospital admissions, selected biochemical analyses, etc., a parallel and synergistic deep phenotyping study will allow us to determine several of the most import early disease manifestations present even prior to any official and often arbitrary cardiometabolic disease diagnosis criteria. Accordingly, the complimentary study approaches will enable us to quantify the extent to which the T2D associated co-morbidities coronary arteriosclerosis, cardiac autonomic neuropathy, coronary calcification, NAFLD, retinopathy, and diabetic kidney disease are present among elderly people without T2D and with a known normal glucose tolerance for two decades. The existing comprehensive genetic, birthweight, and lifestyle information, collected and analyzed over 20 years, provides unparalleled opportunities to determine how individual or groups of risk factors affect the natural history of overt and/or preclinical disease manifestations during the most relevant age window with the highest occurrence rates of T2D and associated cardiometabolic diseases. The complementary data most importantly increases our signal

to noise ratio, and thus statistical power, to detect the relative contribution of a variety of distinct risk factors. For instance, having near complete GWAS data allows adjustment for putative genetic confounders influencing associations between birthweight and overt or preclinical disease. All pieces of information with high importance to understand the heterogeneity, and thus for driving, innovating, and implementing precision medicine, of T2D and co-morbidities. Compared with other prospective cardiometabolic studies including the FHS (Framingham Heart Study) and MESA (Multi-Ethnic Study of Atherosclerosis), a unique feature of the Inter99 cohort, is its detailed assessment of glucose tolerance with standard 75-gram oral glucose tolerance tests in all participants at the baseline examinations, as well as our broader focus on diabetes related cardiometabolic outcome variables including assessments of subclinical diabetes related disease manifestations in arteries, liver, eye, kidney, and nerves at the 20-years follow-up examinations.

The major etiological factors underlying risk of T2D, and its co-morbidities include genetics on one side, and pre- and postnatal environmental exposures on the other (**Box 1**). As for risk factors in pregnancy, the remarkably most accurate marker predicting cardiometabolic disease is weight at the time of birth [65,66]. While there has been much focus on identifying more specific exposures underlying the association between LBW and disease risk, no single factor influencing fetal growth during pregnancy has yet been identified to explain the association. In contrast, multiple epidemiological and animal studies have documented that virtually all factors in pregnancy that negatively influence fetal growth and birthweight including maternal smoking, diet, and energy intake, reduced placental blood flow etc. are associated with increased risk of cardiometabolic diseases in the offspring [67]. Accordingly, rather than representing only a risk marker, LBW may represent a mediator of the totality of adverse events and lifestyle factors in pregnancy that influence later risk of cardiometabolic diseases in the offspring, justifying birthweight as the so far unparalleled cardiometabolic risk marker of prenatal disease exposures.

Based on the Inter99 baseline health examinations, we previously confirmed LBW to be associated with T2D prevalence at a mean age of only 46 years [35]. As the first 20-year follow-up initiative, we studied the association between birthweight and T2D incidence rates [68]. Using the Danish registries, birth records of 4,590 Inter99 participants were linked with age at T2D diagnosis, as well as relevant covariates. We identified 492 new T2D cases since 1999, and subsequently documented that T2D incidence rate decreased with increasing birthweight in a surprisingly linear manner [68]. Interestingly, our study clearly supported the notion that the other major etiological factors of genetics and obesity appeared to operate as independent and most likely additive risk factors on top of that of lower birthweight [68].

Further comprehensive registry analyses of the full range of T2D vascular complications and co-morbidities will provide unparalleled insights into previously unrecognized differential T2D and co-morbidity sub-phenotypes and their underlying etiologies. Here, we for example can determine the extent to which T2D patients with the lowest birthweights may be characterized by a more severe clinical presentation as recently suggested [13]. To improve our understanding of T2D and its sub-phenotypes, similar analytical strategies will be applied for the various early disease markers and manifestations determined in the deep phenotyping clinical follow-up study.

The extended InterDAG subgroup study is aiming to better understand (and adjust for) the impact of diet, whole body sodium and potassium balance, as well as physical activity, on diurnal glucose levels

and fluctuations across a wide range of the glucose tolerance spectra. Blood samples available from the baseline health examinations, along with samples from the reexaminations, will be available for extended micronutrient and multi-omics analyses including whole genome sequencing, metabolomics, lipidomics, transcriptomics, epigenomics, proteomics, and metagenomics. Our vision includes extensive application of AI based analyses to integrate the clinical, biochemical, and genetic data over time, across and beyond current clinical diagnostic cardiometabolic disease criteria.

The Inter99 20-year follow-up study furthermore provides a unique opportunity to study age-related outcomes, such as sarcopenia and physical function. It is well established that lifestyle (diet, physical activity, smoking and BMI) influences the risk of chronic disease, thus Inter99 20-year data will allow for the study of long-term impact of lifestyle in early adulthood on subsequent age-related disease manifestations. These data will for instance allow us to study the trends in dietary habits and physical activity patterns and their impact on muscle strength and function in middle- and old age in people with and without T2D or CVD. As such, our Inter99 follow-up study will provide important insights into the mechanisms underlying age-related processes in both healthy and diseased individuals.

An inherent limitation of the study is its observational nature that does not allow us to make strong inferences about causality. Further limitations include the fact that all individuals participated in screening for CVD risk and a personalized lifestyle intervention program from 1999 up to five years thereafter, as well as the likelihood that only the healthiest cohort participants may show up for the follow-up examinations, both potentially limiting the generalizability of our findings. Finally, nearly all participants are of Danish ethnicity, as Danish literacy was a prerequisite at baseline inclusion.

In conclusion, the current combined epidemiological registry and deep phenotyping 20-year clinical follow-up study provides an example of the value of reexamining an existing and already extensively characterized T2D and cardiometabolic cohort, with the overall aim to better understand etiologically distinct disease trajectories and sub-phenotypes. This will facilitate development of better and more efficacious precision medicine prediction, clinical care, as well as overall treatment approaches in T2D and associated diseases. The cohort data will via a scientific steering group be available for international collaborations.

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Contributors

KB and CB drafted the first version of the manuscript. AV and AL initiated the study. KB, CB, MA, FBK, CFBN, BL, RW, CS, KN, NRJ, CSU, MK, NG, JK, LML, LK, ALM, KFK, RL, TH, AL, AV

designed and managed the study. All authors critically reviewed the manuscript, read, and approved the final version of the manuscript.

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Competing interests

None declared.

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Figure legends

Figure 1. Flow chart of participation in the Inter99 study, 1999 – 2023. The dark blue column represents the Intervention group (A+B) where 52% of the invited had a baseline examination performed in 1999. The eligible participants for the 20-year follow-up study are recruited among these individuals. The light-blue columns indicate individuals followed by questionnaires (C) and the reference population (D) who are not recruited for the 20-year follow-up examinations.

Figure 2. Overview of the 20-year deep-phenotyping follow-up examinations and possibilities of coupling to baseline and register data as well as for future and extended analyses

Table 1. Summary of data collected at the Inter99 baseline and the 20-year follow-up study (including extended sub-studies)

Data	Variables	Baseline	20-year
Questionnaire-based information			
Demographics	Sex, age, family, marital status, education, employment status, household income	X	X
Diseases	Chronic diseases, contact to health care system, symptoms	X	X
Health	Self-rated health, stress, sleep	X	X
Lifestyle	Physical activity, smoking, alcohol, diet, network	X	X
Deep-phenotyping health assessment			
Anthropometry	Height, weight, waist, and hip circumference	X	X
Bioelectrical impedance	Fat and lean (muscle) body mass		X
Blood pressure	Systolic and diastolic blood pressure, resting heart rate	X	X
Cardiac autonomic neuropathy	Resting heart rate variability and cardiovascular autonomic reflex tests		X
Cardiac CT	Coronary atherosclerosis, cardiac chamber size, LV hypertrophy		X
Continuous glucose monitoring	7-day 24-hour glucose levels		X(S)
Dynamometer and sit-to-stand test	Muscle strength		X
Electrocardiography	ECG-intervals, -amplitudes and diagnostic statements	X	X
Ophthalmic examination	Ocular fundus characteristics, retinopathy	X(S)	X
Oxymeter	Oxygen saturation		X
Pulse wave velocity	Arterial stiffness		X
Spirometry	Lung function	X	X(S)
Transient elastography	Liver stiffness and steatosis		X
Laboratory assessments			
Blood biochemistry	Leukocytes and differential count, thrombocytes, electrolytes (sodium, potassium, calcium), glucose, HbA1c, lipids (total cholesterol, HDL, LDL, VLDL, triglycerides), kidney function (creatinine, eGFR, urea, albumin), Vitamin K status (dephosphorylated-uncarboxylated matrix-gla Protein), liver function (ALAT, ASAT)	X	X
Urine biochemistry	Albumin, creatinine, sodium, potassium	X	X
Biobanking (-80°C)			
Blood	Fasting blood samples (whole-blood, serum, and plasma)	X	X
Urine	Spot urine 24-hour urine collection	X X(S)	X X(S)

Feces	Fecal samples		X(S)

(S) Subgroup of participant.

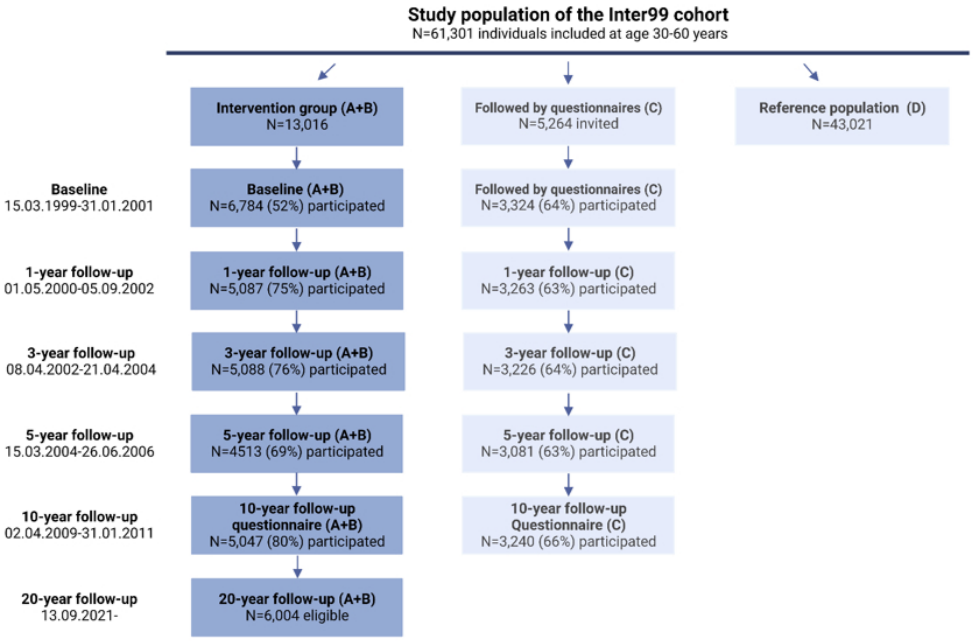

Figure 1. Flow chart of participation in the Inter99 study 1999 – 2023. The dark blue column represents the Intervention group (A+B) where 52% of the invited had a baseline examination performed in 1999. The eligible participants for the 20-year follow-up study are recruited among these individuals. The light-blue columns indicate individuals followed by questionnaires (C) and the reference population (D) who are not recruited for the 20-year follow-up examinations.

573x407mm (38 x 38 DPI)

Figure 2.

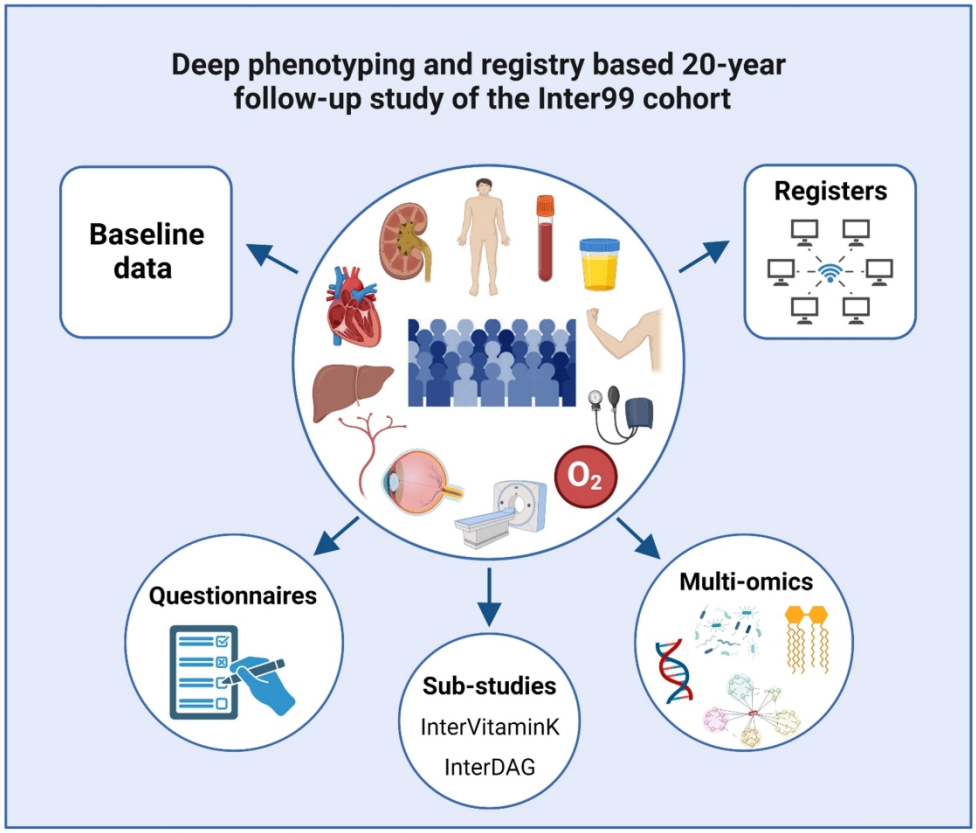

Figure 2. Overview of the 20-year deep-phenotyping follow-up clinical examinations, the possibilities of coupling to baseline and register data as well as for future and extended analyses.

183x167mm (330 x 330 DPI)

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℃ 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℃ 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, Canon 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 chair stand)

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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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5-6
Objectives	3	State specific objectives, including any prespecified hypotheses	5-6
Methods			
Study design	4	Present key elements of study design early in the paper	5-7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6-7
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6-7
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	-
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7-9
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	7-9
Bias	9	Describe any efforts to address potential sources of bias	5
Study size	10	Explain how the study size was arrived at	7
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	10-11
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	10-11
		(*b*) Describe any methods used to examine subgroups and interactions	10-11
		(*c*) Explain how missing data were addressed	10
		(*d*) If applicable, explain how loss to follow-up was addressed	10
		(*e*) Describe any sensitivity analyses	-
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	6-7
		(b) Give reasons for non-participation at each stage	n/a
		(c) Consider use of a flow diagram	n/a
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	-
		(b) Indicate number of participants with missing data for each variable of interest	-
		(c) Summarise follow-up time (eg, average and total amount)	7
Outcome data	15*	Report numbers of outcome events or summary measures over time	-
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	-
		(*b*) Report category boundaries when continuous variables were categorized	-
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	-
Discussion			
Key results	18	Summarise key results with reference to study objectives	-
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	-
Generalisability	21	Discuss the generalisability (external validity) of the study results	-
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.