Research Protocol

Deep Phenotyping of Bone Disease in Type 2 Diabetes and Relations to Diabetic Neuropathy

Ansøgning v/ Regional Videnskabsetisk Komité:
Signed:

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List of abbreviations

aBMD	Areal bone mass density
AGE	Advanced glycation end products
ALAT	Alanin-aminotransaminase
AP	Anterior-Posterior
BI	Business intelligence
BMD	Bone mineral density
BMI	Body mass index
BMSi	Bone Material Strength index
CAN	Cardiac autonomic neuropathy
CDT	Cold detection threshold
CI	Confidence Interval
CoP	Center of pressure
COPD	Chronic Obstructive Pulmonary Disease
CST	Chair Stand Test
CT	Computer Tomography
DD2	Danish Center for Strategic Research in Type 2 Diabetes
DM	Diabetes Mellitus
DPN	Diabetic Polyneuropathy Neuropathy
DXA	Dual-energy X-ray absorptiometry
ECG	Electrocardiogram
EDTA	EthyleneDiamineTetraAcetic acid
eGFR	estimated Glomerular Filtration Rate
EPJ	Electronic Patient Journal
GABA	γ-aminobutyric acid
GDPR	General Data Protection Regulation
GGT	Gamma-Glutamyltransferase
GLP-1	Glucagon Like Peptide 1
Gy	Grays
HbA1c	Hemoglobin A1c
HR-pQCT	High-resolution peripheral quantitative computed tomography

ICH GCP	Harmonised Tripartite Guideline for Good Clinical Practice
IGF-1	Insulin-like growth factor-1
IC	Informed consent
IENF	Intra epidermal nerve fibers
IQC	International Quality Control
MARD	Mild age-related diabetes
ML	Medial-lateral
MNSI	Michigan Neuropathy Screening Instrument
MOD	Mild obesity-related diabetes
N	Sample requisition numbered
NBV	National treatment guidelines in Endocrinology
OCT	Optical Coherence Tomography
PTT	Perception threshold tracking
QST	Quantitative sensory testing
SAF	Skin autofluorescence
SGLT-2	Sodium-Glucose Cotransporter-2
SIDD	Severe insulin-deficient diabetes
SIRD	Severe insulin-resistant diabetes
Sv	Sieverts
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TBS	Trabecular bone score
TZD	Thiazolidinedione
TUG	Timed Up and Go
UGS	Usual gait speed
vBMD	Volumetric bone mineral density
VFA	Vertebral Fracture Assessment
VPT	Vibration Perception Threshold
WDT	Warm detection threshold
WHO	World Health Organization

2. BACKGROUND AND SIGNIFICANCE

2.1. Background

Type 2 diabetes (T2D) is a prevalent disease currently affecting 422 million people worldwide (2014), leading to a global prevalence of 8.5 % among adults[1]. The total number of people living with diabetes (T1D and T2D) is projected to rise to 783 million by 2045[2]. Thus, prevention and intervention of complications is important. A less well-known complication of T2D is diabetic bone disease. T2D is associated with an normal or even increased bone mineral density (BMD)[3,4], which in theory should lead to a decreased risk of fractures. However, T2D is associated with an increased risk of any fractures, with relative risks reported up to 1.7[5–8]. A number of meta-analyses reports an 1.08 to 1.7-fold increased risk of hip fractures in patients with T2D[3,5–7,9,10]. Evidence of vertebral fractures is more ambiguous, with the meta-analysis by Moayeri et al.[7] reporting an increased risk, while Jia et al.[6] did not find an significantly increased risk of vertebral fractures in T2D. Another meta-analysis by Koromani et al.[11] showed a lower risk of prevalent but increased risk of incident vertebral fractures in T2D. Furthermore, there seems to be an increased risk of fractures at more distal sites such as the humerus[8,12,13], distal forearm[12], ancle[8] and foot[7] but the evidence is more limited. The reason for this increased fracture risk is not fully understood, but may result from a combination of a decreased bone biomechanical competence and an increased risk of falls[14]. Several associations thus need more detailed examination and characterization in the relationship between T2D and skeletal health.

2.2. State of the art

The increased fracture burden in diabetes might partially be explained by an increased risk of falls and by alteration in the fall mechanism. A meta-analysis by Yang et. al. [15] showed that older persons with diabetes has increased risk of falls compared with healthy controls. Several risk factors that contribute to falls in diabetes exists. These include impaired postural control and gait, diabetic neuropathy, impaired vision, vestibular disorder, cardiovascular disease, comorbidity including hypertension, drugs and hypoglycemia[16]. Furthermore, patients with T2D has a higher risk of sarcopenia[17] and decreased muscle function[18], which has been proposed as a contributor to increased fall tendency[19] and increased risk of fractures[20]. Several studies of diabetes and fracture risk have included data on falls. In these studies, higher fracture risk associated with T2D persisted even after adjustment for increased frequency of falls[21–23]. This suggest that additional underlying mechanisms such as decreased bone quality is involved in the increased fracture risk in T2D. Bone quality is affected by bone mass, bone turnover and bone material properties.

It is suggested that advanced glycosylation end products (AGEs) produced in response to hyperglycemia are incorporated in the bone structure, hence reducing the material and biomechanical properties of bone by stiffening the bone collagen and reducing bone strength[24]. Furthermore, the hyperglycemia triggers hypermineralization in the bone causing high BMD[25]. The interplay between bone and glucose metabolism is complex and not fully understood. An interaction is present between osteocalcin, which is a bone formation marker, but also interacts with insulin sensitivity, thus forming a bone-beta-cell axis[26,27]. T2D is associated with decreased bone turnover caused by osteocyte dysfunction[28,29]. It has been suggested that the low bone turnover in T2D cause microcracks, thereby increasing fracture risk[30]. Bone Material Strength index (BMSi) is measured by microindentation and is reported to be decreased in T2D[31,32], which is considered to reflect decreased ability of bone to resist microcrack generation and propagation[33].

Alterations in bone microarchitecture may be present among T2D patients, resulting in an increased fracture risk. Trabecular bone score (TBS), a textural parameter that can be applied to DXA, has been reported to be significantly decreased in patients with T2D in a recent meta-analysis[34]. TBS may even be decreased in prediabetes, indicating that the degradation of bone microarchitecture may occur in early stages of the disease[35]. Higher HbA1c levels might be related to lower TBS values[35–38], as well as the presence of microvascular disease[39]. Additionally, low TBS values has been associated with adiposity (estimated by the relative fat mass) and insulin resistance[40], and one study found that reduction of visceral fat could be related to improvement of TBS[41]. High resolution peripheral quantitative computed tomography (HRpQCT) is a non-invasive 3D imaging modality that permits the assessment of bone microarchitecture, including the measurement of volumetric cortical and trabecular bone mineral density (vBMD) and cortical thickness/porosity, bone strength as well as other parameters in the peripheral skeleton such as distal radius and tibia[42]. In some studies, but not all, cortical porosity is reported to be higher in T2D compared to controls in postmenopausal women[43–45]. Furthermore, T2D patients are reported to have lower cortical vBMD and cortical thickness[45,46]. The changes in microarchitecture results in a decreased bone quality, and this decreased peripheral bone quality might attribute to fractures in T2D.

Shanbhouge et al.[45] found that the observed cortical deficits were a characteristic of the T2D patients with microvascular complications rather than all T2D patients. However, the study was not able to perform an adequate assessment of the separate roles and severity of neuropathy, retinopathy, and nephropathy in bone structure due to limited sample size. A meta-analysis by Liu et al.[47] reported that T2D patients with diabetic neuropathy have an increased risk of developing osteoporosis and fragility fractures with an OR of 2.15 (95% CI 1.56-2.97). Contrastingly, a Danish study by Khan et al.[48] using patients enrolled in the Danish Center for Strategic Research in Type 2 Diabetes (DD2) cohort were not able to find a significant association between diabetic neuropathy and fractures. However, they did find an increased risk of falling. A well-powered retrospective study by Lee et. al[49] found that older male Veterans with diabetes (>98% had T2D) have an increased risk of any fractures and hip fractures, and diabetic neuropathy was reported to be the most important mediator of fracture risk observed in their cohort. A histomorphometry study in humans with high bone turnover showed that the nerve profiles density was 5-fold higher in the intracortical pores compared to bone marrow and periosteum, and the authors suggest an anatomical link between innervation and bone remodeling[50]. Overall, there seems to be a higher fracture risk in subjects with T2D and diabetic neuropathy, which is a very common complication of T2D. However, the exact mechanisms behind this remain to be elucidated.

Multiple studies have investigated the effects of antidiabetic medication on fracture risk. Several studies found an association on glitazones and increased fracture risk[51–53]. Evidence of the effect on insulin on fracture risk in T2D is more conflicting, as insulin use have shown both increased[13,54,55], neutral[56] and decreased[57] outcome on fracture risk. Also sulphonylureas are reported to have both neutral[53,58,59], negative[60,61], or perhaps even protective[57] effect on fractures risk. Other medications have been associated with a decreased or neutral effect on the risk of fractures in T2D, such as Metformin[51,53,57,58], DDP-4 inhibitors[56,62,63], GLP-1 receptor agonists[62,64] and SGLT-2 inhibitors[65,66]. In addition, concomitant medications related to comorbidities might also play a role in fracture risk in T2D. Thiazides have been associated with a decreased fracture risk, while loop diuretics are associated with higher fracture risk[67]. An updated meta-analysis revealed no significant effect of statin treatment on the risk of fractures[68]. Also drugs commonly

used for diabetic neuropathy such as tricyclic antidepressants[69,70] and γ-aminobutyric acid (GABA) analogs[69] are associated with increased risk of falls and fracture.

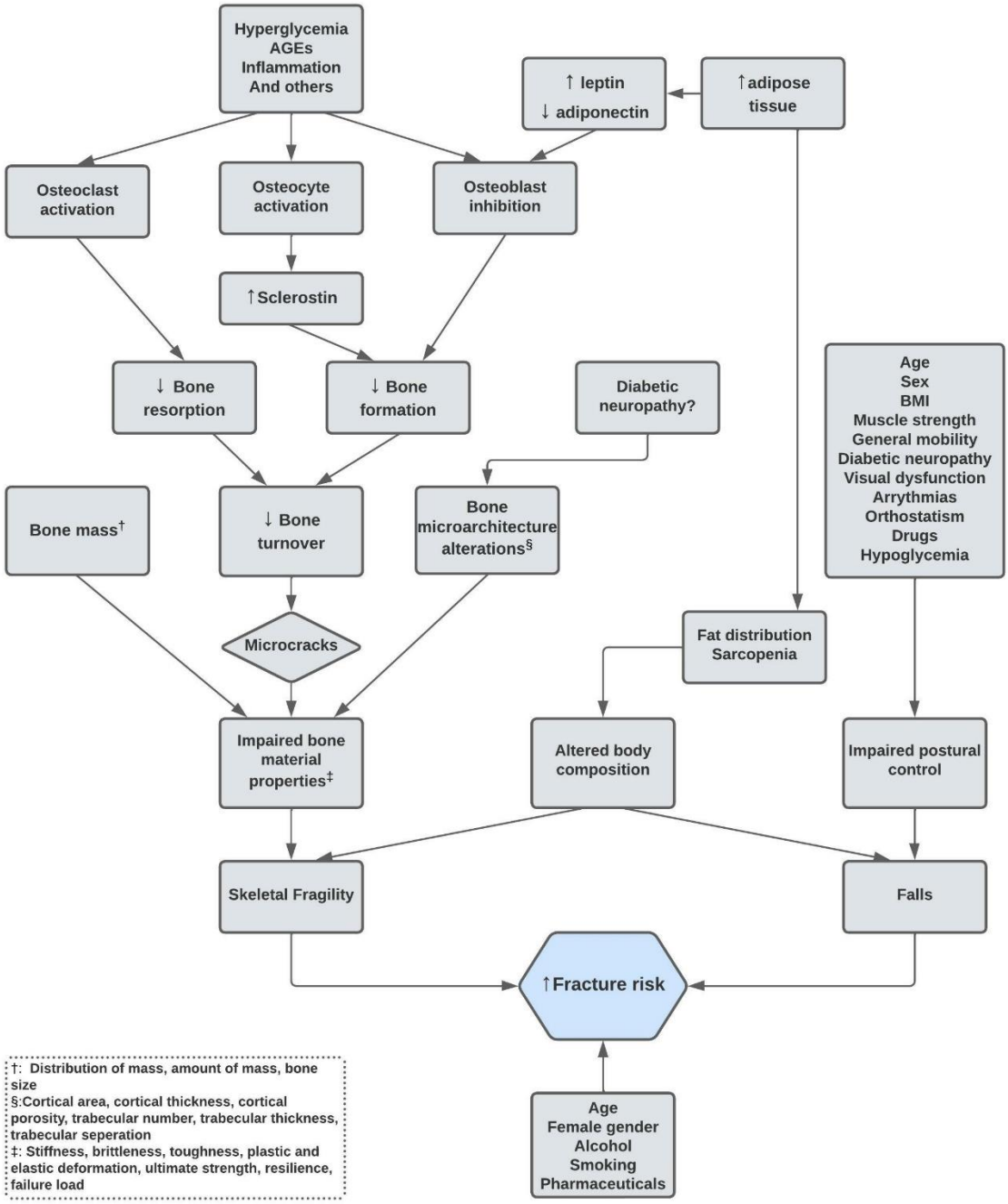

Figure 1: Factors leading to increased fracture risk in T2D

2.3. Significance and perspectives of the project to the research area

Osteoporosis is a major public health problem. Hip fractures are associated with increased mortality[71,72], and the risk is even higher in diabetic patients compared to non-diabetics[73]. The cost of osteoporosis in the EU in 2010 was estimated at €37 billion with the highest estimated cost per capita in Denmark. Thus, osteoporosis

has major economic consequences for society[74]. As the risk of fractures will increase with the expected increasing prevalence of T2D, it is important to identify risk factors and mechanisms underlying diabetic bone disease and fracture risk to enhance treatments and prevention.

This study will provide new knowledge on T2D bone phenotyping and the crosstalk between bone, nerves, muscle, fat and beta cells. Furthermore, the study contributes with a characterization of the interplay between diabetic neuropathy and bone disease which could play a pivotal role in advancing the field of neuroskeletal biology.

The study will include novel and advanced technologies in the assessment of the T2D bone phenotype covering bone microarchitecture, bone strength and bone remodeling markers. In addition, the study will include comprehensive functional assessments spanning from motor to neuronal assessments with inclusion of sensory assays for both large and small fibers and evaluation of autonomic function.

3. STUDY OBJECTIVES

We hypothesize that a crosstalk between beta cells, bone-, nerve-, muscle- and fat tissues exists, and our main goal is to attain a better understanding of these relations and describe the T2D bone phenotype.

Hence, our aims are to:

- Compare bone microarchitecture, bone biomechanical competence, and bone turnover markers in T2D with and without fractures.
- Examine how different phenotypes of diabetic neuropathy affects bone microarchitecture, bone material strength and bone turnover markers as well as postural control and physical function.
- To examine how insulin resistance effects bone microarchitecture, bone material strength and bone turnover.
- To examine the relationship between T2D phenotype (classical/insulinopenic/hyperinsulinemic or SIDD [severe insulin deficient diabetes]/SIRD [severe insulin resistant diabetes]/MOD [mild obesity-related diabetes]/MARD [mild age-related diabetes]) and body composition (fat and muscle mass and fat distribution, i.e. visceral adipose tissue)
- To investigate how microvascular complications (neuropathy, retinopathy, and nephropathy) are affected by T2D phenotype (classical/insulinopenic/hyperinsulinemic or SIDD/SIRD/MOD/MARD)

4. METHODS

4.1. Study Design

Cross sectional study.

4.2. Study population

The study strives to include a total of 300 subjects with T2D divided into three well-defined groups:

- T2D F-/N-: Subjects with T2D and no previous history of any fractures or diabetic neuropathy (*n*=160)
- T2D F+: Subjects with T2D with a previous history of a fracture(s) (any fracture, major osteoporotic fracture (MOF) and peripheral) (*n*=100)
- T2D N+: Subjects with T2D matched by age and sex with severe peripheral (vibration perception threshold (VPT) > 50) or a history of autonomic neuropathy (*n*=40).

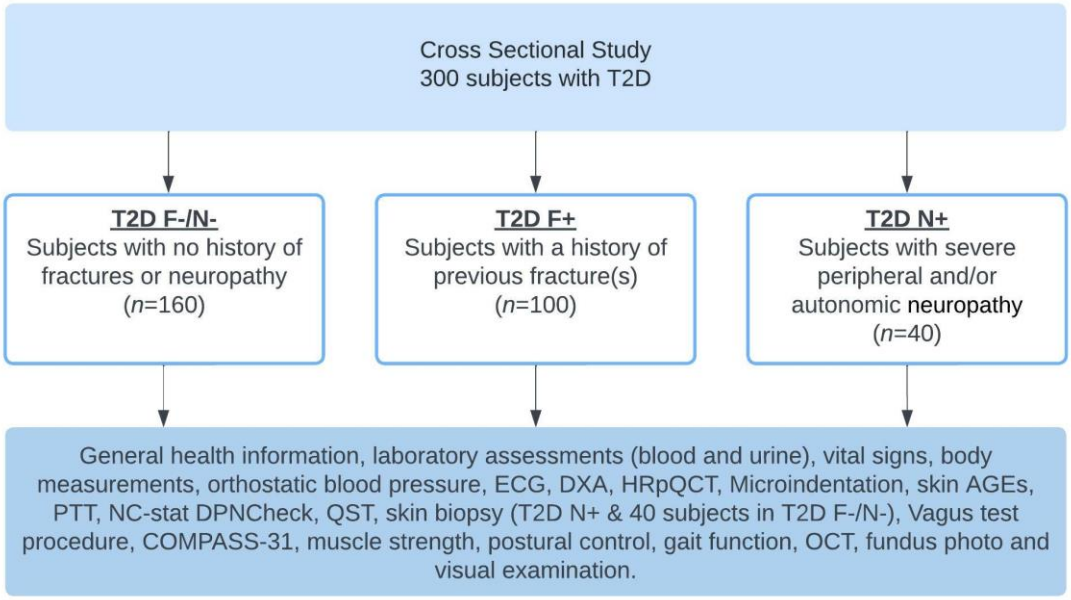

Figure 2: Study design

Inclusion criteria

1. Men and women with minimum 40 years of age.
2. Diagnosis of T2D. At least one of the following criteria must be met at diagnosis:
    a. HbA1c ≥ 48 mmol/mol (6,5 %)
    b. Plasma glucose ≥ 11,1 mmol/l
    c. Fasting plasma glucose ≥7,0 mmol/l

    Clinical effect of oral antidiabetic medication strengthens the diagnosis.
3. Diagnosis of diabetes at least one year prior to inclusion of the study.
4. A history of fracture(s) (confirmed by radiographs analyzed by radiologist) following the diabetes diagnosis (T2D F+ group)
5. Diagnosed with severe peripheral (VPT ≥ 50) or autonomic neuropathy defined by cardiac autonomic reflex tests or severe abnormalities in orthostatic blood pressure (T2D N+ group)
6. Signed the informed consent.
7. Not defined by the exclusion criteria.

Exclusion criteria

1. Severe decreased liver function (Alanin amino-transaminase (ALAT) >250 U/l, Gamma-Glutamyltransferase (GGT) >150 U/l).
2. Moderate to severe kidney dysfunction, estimated Glomerular Filtration Rate (eGFR) <15 mmol/L/1,73m^2.
3. Pregnancy or breast feeding.
4. Active malignancy or terminal ill.
5. Previous chemotherapy or immunomodulating treatment
6. Known severe vitamin deficiency

7. Current or previous alcohol- or drug abuse.
8. Not being able to understand Danish written and/or verbally.
9. Terms according to investigators judgement that makes subjects unsuitable to participate including lack of ability to understand and comply with instructions and/or reduced physical ability, limiting the ability to participate in the examinations.
10. Participating in other clinical studies utilizing experimental treatment or medication.
11. Subjects with pathologic fractures (defined as fractures due to local tumors, tumor-like lesions, or focal demineralization as visualized on radiographs).
12. Primary hyperparathyroidism, Paget's disease and other metabolic bone diseases, uncontrolled thyrotoxicosis, celiac disease not controlled by diet, known hypogonadism, severe COPD, hypopituitarism, Cushing's disease.
13. Fracture < 6 month ago
14. Initiation of antiresorptive or bone anabolic drugs <12 months ago to ensure stable bone turnover markers.
15. History of fractures following the diagnosis of diabetes (T2D F-/N- and T2D N+ groups).
16. History of peripheral or autonomic neuropathy defined by cardiac autonomic reflex tests or severe abnormalities in orthostatic blood pressure (T2D F-/N- group).

4.3. Assessment of resources

The study will be conducted in collaboration with the department of Endocrinology and Steno Diabetes Center North Jutland, which has access to subjects with diabetes and can provide the necessary premises.

The department of Endocrinology has a large laboratory with 7 Bio. Med. Lab. Technologists dedicated for research. We have extensive experience with DXA technology and highly skilled technicians with years of experience. An extensive International Quality Control (IQC) program according to the guidelines of the Society for Clinical Densitometry is in place for the DXA scanners.
The laboratory offers blood sampling facilities as well as facilities for blood testing including state of the art validated bone turnover markers. A collaboration with the department of Clinical Chemistry also exists.

4.4. Recruitment

Recruitment will be targeted at subjects with T2D in the Region of North Denmark and can include both T2D patients in general and participants in the DD2 study (https://dd2.dk/). Recruitment of participants will be based on advertisements in media, e.g. on www.forsoegsperson.dk, social medias as Facebook and Instagram, local medias and papers. Patients will also be recruited from the outpatient clinics of the department of Endocrinology, Aalborg University Hospital in Aalborg and Farsø as well as North Denmark Regional Hospital in Hjørring. Lists of patients from the BI (Business intelligence) Unit at the Region of North Denmark will be used for direct mailing via secure mail pending approval from the competent bodies at the Region of North Denmark. An existing database where participants have given written consent (within the last year) to be contacted regarding research projects may also be used. This database is created and administrated by the North Denmark Region and approved by its legal staff. Potential participants are required to use a secure access (MitID), and if they do not actively renew their consent within a year, it is

automatically withdrawn. We will hang posters and hand out flyers at general practitioners, podiatrists, supermarkets and training centers at places flyers are allowed to hang.
The healthcare professionals at Aalborg University Hospital, regional hospitals and home-care services in the North Jutland Region will be contacted and asked to be involved in the patient recruitment.

4.5. Study procedures

General health data from medical records and interview

To enable the best possible adjustment of confounding factors in later analysis, general health information for each subject will be assessed through medical records and interviews. These include assessment of weekly alcohol consumption, daily tobacco use, daily activity level (at work and at home), comorbidities, signs and symptoms of neuropathy, familiar disposition to osteoporosis, risk factors/comorbidities associated with osteoporosis in accordance with the National treatment guideline in Endocrinology (NBV), prior biochemistry, duration of diabetes, prior fractures (localization, high/and medication use (including hormone therapy such as oral contraceptives etc.) for at least one month priory to baseline measurements.

Laboratory assessments

Individual laboratory values will be compared to their relevant reference range (when existing) and flagged as being below or above the range. The blood samples will be taken and handled as usual by the Research bio. Med. Lab. technologists at Aalborg University Hospital.
If laboratory samples need to be re-done, due to missing results (e.g. hemolysis, sample leaked, inconclusive, lost in transit etc.), the subject should be called in for resampling. Final laboratory reports must be reviewed, dated, and signed by the investigator on the day of evaluation. It must be specified by the investigator whether out of range results are clinically significant. The subjects are allowed to receive or betray results (See informed consent form).

Biochemistry:

The following samples will be taken at the screening visit (total 4 ml):

- Creatinine, eGFR
- ALAT
- Gamma-Glutamyltransferase
- TSH
- 25-hydroxy D-vitamin
- Cobalamin

The following samples will be taken at the study day (total 84 ml including samples for the research biobank):

- HbA1c
- Fasting-C-peptide and fasting P-glucose (for HOMA2-%B and -IR)
- CRP
- Lipids (LDL, HDL, total cholesterol, triglycerides)
- PTH
- Calcium
- Phosphate

- Magnesium
- Carbamide
- Sodium
- Potassium
- Alkaline phosphatase
- Bilirubin
- Albumin
- M-component
-
- Hematology
  - Hemoglobin
  - Erythrocytes
  - Reticulocytes
  - Hematocrit
  - Middle Cell Volume
  - Middle Cell Hemoglobin Concentration
  - Leukocytes and diff. counting
  - Thrombocytes
  - Folate
  - Ferritin
  - Transferrin
- hsCRP*
- FGF23*
- Sclerostin*
- Osteoglycin*
- Osteocalcin*
- Undercarboxylated osteocalcin (ucOC)*
- Osteopontin*
- P1NP*
- CTX*
- EsRAGE*
- GIPR*
- Testosterone/estradiol *
- FSH*
- LH*
- Adiponectin*
- Leptin*
- IGF-1*

*: The sample will be analyzed after study completion from blood stored in the research biobank (seesection 7.2)

Estimated time: 10 min

24-hour urine collection:

- Creatinine
- Carbamide
- Sodium
- Potassium
- Volume
- Protein
- Albumine
- Ratios

Assessment of vital signs and body measurement

Cardiovascular parameters including heart rate, systolic and diastolic blood pressure. This will be assessed using an Electronic Blood Pressure Device (Omron M6 Comfort IT Intelligence) after 5 min of quiet sitting. Blood pressure will be measured three time and the mean value will be calculated of the last two measurements. If the subject is using antihypertensive medication to control blood pressure, then the medication should be taken as usual prior to assessing vital signs.
Height: (Without shoes) will be measured and rounded to the nearest half centimeter.
Body weight: Should be measured in kilograms (kg) without overcoat and shoes and wearing only light clothing. Body weight will be recorded to one decimal place.
BMI is calculated.

Estimated time: 10 min.

Orthostatic blood pressure

After the normal blood pressure procedure, the subjects will be asked to stand for three minutes and afterwards a new blood pressure will be measured.
Orthostatic hypotension is defined as a decrease in systolic blood pressure of 20 mmHg or a decrease in diastolic blood pressure of 10 mmHg within three minutes of quiet standing when compared with blood pressure from the sitting position.

Estimated time: 5 min.

12 lead-Electrocardiogram

An ECG-12 lead will be performed in a lying position and the subject will have bare chest . The ECG must be interpreted by the investigator and documented.
The evaluation must follow the categories:

- Normal
- Anormal
    - Clinically significant? (Yes/no)

ECG findings will be summarized descriptively. If an ECG-12 lead has already been performed within three weeks before entering the trial and if the results are available, the procedure does not need to be repeated. However, if clinical warranted as judged by the investigator the ECG-12 lead should be repeated.

Estimated time: 10 min.

DXA scan

Will provide BMD at the hip and spine, TBS (trabecular bone score) and body composition measures (lean body mass, fat mass and distribution, muscle mass).

Overview

DXA will be used to assess overall skeletal changes that often occur with age by measuring bone mineral content (BMC) and bone mineral density (BMD). In addition, total body fat and lean muscle mass measurements can give insight into the influence of age, sex, and race/ethnicity on the skeleton relative to these measures. DXA measurements can be used to determine the prevalence of osteopenia and osteoporosis. DXA technology has evolved from pencil beam to fan beam, allowing short acquisition time and improved image quality. In clinical practice, 'areal' bone mineral density (aBMD; g/cm^2) assessment of lumbar spine (L1–L4), proximal femur (femoral neck and total hip) and forearm (distal) is made by central DXA. Interpretation of aBMD measurements is based on the World Health Organization (WHO) recommendations. Osteoporosis can be diagnosed if the value of aBMD is 2.5 or more standard deviations (SD) below the mean value of a young reference population (T score ≤−2.5). Central DXA can also provide whole-body imaging for total and regional aBMD, body composition (lean muscle and fat mass) and Vertebral Fracture Assessment (VFA). Using device-specific thresholds peripheral DXA may play a role in identifying those at risk of osteoporotic fracture, especially when there is limited or no access to central DXA[75].
The greatest increase in the DXA usage has been observed in the use of central densitometry[76]. As with other X-ray-based imaging methods, radiation dose from bone densitometry techniques that use ionizing radiation (DXA) must be kept as low as reasonably achievable.

Radiation dose

Various radiation dose parameters are used in diagnostic radiology, the most commonly being absorbed dose and effective dose. Absorbed dose, expressed in Grays (Gy), is a measure of the energy per unit of mass deposited in the tissue and organs of the body. Radiation dose from ionizing radiation is frequently quantified in terms of the effective dose. The effective dose, expressed in Sieverts (Sv), is calculated from information about absorbed doses to the organ or tissue exposed to X-rays and the relative radiation risk assigned to each of these organs or tissues. The effective dose is a useful quantity for comparison among different sources of ionizing radiation, such as that from DXA and natural background radiation. The worldwide average effective dose from natural background radiation is 2.4 mSv/year.
Whole-body DXA is an established procedure for the assessment of skeletal mineral status of the whole body and the measurement of body composition. Effective doses for whole-body DXA examinations varies and were found to be between 0.001 and 0.01 mSv. Comparable with chest-x ray 0.01mSv and dental x-ray 0,005mSv a low dose.
We plan to perform Whole Body and central DXA scan to all subjects. Pregnancy status will be assessed on all fertile females. If the result of the pregnancy test is positive, the sampled participant will be excluded from the entire study. Participants will receive a maximum of three DXA scans (whole body DXA scan, AP spine scan and femur scan). Under no circumstances should a whole-body scan be repeated. If the subject is recently scanned at Aalborg University Hospital within 6 months, there is no need for additional scan.

Personnel

Health technologists who are certified Bio. Med. Lab. Technologists will conduct all DXA scans.

Estimated time: 45 min.

HRpQCT

Bone structure is an integral determinant of bone strength. The availability of HR-pQCT has made it possible to measure three-dimensional bone microarchitecture and volumetric bone mineral density in cortical and trabecular compartments of the distal radius and distal tibia, with accuracy previously unachievable and with relatively low-dose radiation.
Analogously to DXA, radiation exposure from HRpQCT is low (0.01 mSv), although slightly higher if compared with DXA[77].

The scanner's gantry is relatively narrow and shallow (rear physical stop) only allowing the distal peripheral skeleton to be accommodated. The limb being scanned is immobilized in a carbon fiber shell. A scout view, essentially a two-dimensional x-ray scan, is obtained so that the operator can identify a precise region for the three-dimensional measurement. Because HR-pQCT uses a polychromatic X-ray source it is subject to beam hardening as well as scatter artefacts, which can significantly impact geometric and densitometric measures. Once the images have been acquired, a default patient evaluation protocol is used to analyze the scans over the entire 9.02 mm three-dimensional region to assess a wide range of standard and optional structural and density parameters.
HR-pQCT has been demonstrated to be able to discern between women and men with and without fractures. The inherent strength of HR-pQCT is its ability to assess a large slice of bone in three-dimensions for differences in cortical and trabecular characteristics and to identify those characteristics that are most associated with bone weakness, by FEA bone strength modelling. When compared with DXA aBMD, HR-pQCT measures have a better discriminatory ability to discern between women with and without fractures

Estimated time: 20-25 min.

Microindentation

Using OsteoProbe®, we will by microindentation measure the material strength index (BMSi), a measure of the hardness and stiffness of the cortical bone in the tibia. The participant will be positioned in supine position with the leg to be measured rotated to orient the flat surface of the medial tibia diaphysis. The mid distance between the medial malleolus and the medial border of the tibia plateau are marked. Following a disinfection of the area using chlorhexidine, Lidocaine is administered by inserting a syringe both subcutaneously and in the periosteal surface. A sterile probe is then inserted at the marked mid-diaphysis, penetrating the skin and periosteum until reaching the bone cortex. While maintaining probe contact with the bone surface, as well as orienting the probe perpendicularly to the tibia surface, the outer housing of the device is slid forward to the subject's leg to initiate a measurement. After the first measurement, the probe

is moved to a new location, at least 2 mm from the prior measurement, to obtain another measurement. At least 8 and maximum 18 indentations are performed on each subject.

Estimated time: 20 min.

Skin AGE Reader

All subjects will be tested with the AGE Reader (Diagnoptics®, Groningen, The Netherlands), which is a non-invasive device that uses ultra-violet light to excite autofluorescence in human tissue. The skin autofluorescence (SAF) is strongly correlated with AGEs. AGEs bind to type 1 collagen in both skin and bone. A study showed that levels of SAF was inversely associated with bone material strength, as confirmed by microindentation[31]. Thus, measuring AGE levels by means of SAF might be a reflection of bone AGEs and a potential biomarker of bone strength and fracture risk. The AGE Reader is validated in subjects with UV reflection on >6% corresponding to Fitzpatrick skin phototypes I-IV.

Estimated time: 5 min.

Assessment of Neuropathy

Perception Threshold Tracking (PTT)

Perception Threshold Tracking is a potential new method for early detection of neuropathy. It is a further development of conventional threshold tracking that excels in describing membrane potential of not only large, but also small nerve fibers. The trial is conducted using two types of surface electrodes placed on the dorsum of the foot: patch and pin electrodes for stimulation of large and small nerve fibers respectively. The perception threshold is then estimated by slowly increasing the intensity of stimuli until the subject presses a button indicating that the stimuli is perceived. As this happens, the intensity is initially heighted by 20 % and then lowered until the subject relieves the button indicating that the stimulus is no longer felt. The intensity is then lowered by 20% and then increased until perception is indicated by pressing the button. This is repeated three to five times to increase precision. The perception threshold is taken as the average of the six to ten times the subject pressed the button. This procedure will be repeated several times using different electrodes. The subject will feel no significant pain, as the stimuli given is just around the individual perception threshold, which is a huge benefit of this method compared to other available neural tests. There will be no risk of burns or injury to the skin, as the stimulator is limited to supplying electricity well below any dangerous level.

Estimated time: 10-15 min.

NC-stat DPNCheck

Gel is applied to the apparatus and the one-time electrode is connected. The device is then placed following the sural nerve from just behind the malleus and up the back of the leg. Using a short electrical stimulation, the device records the nerve conduction velocity and amplitude.

Estimated time: 5 min.

Skin biopsy

Skin biopsy will be performed on the 40 subjects in the T2D N+ group and 40 subjects in the T2D F-/N- group. After ensuring sufficient blood flow the skin is anesthetized using 0,5-1 ml Lidocaine. After 3-5 minutes it is ensured that the skin is indeed numb. After confirming this, two punch biopsies are taken just above the

ankle. The biopsies will be taken using a disposable 3-mm punch under usual sterile conditions. Potential bleeding is stopped by compression and the wound is bandaged as per normal clinical procedure. Before the subject leaves, it is ensured that the bleeding has stopped. Participants are informed to keep the wound dry for 2 days and to change the bandages if needed.

Skin biopsies will be analyzed at the Danish Pain Research Center, Aarhus University. Sections will be stained using the free-floating protocol, and IENF quantified following available guidelines. Additionally, regenerative-related antibodies will be used to identify potential biomarkers of early onset neuropathy. The antibodies used will include primarily PGP and GAP-43.Estimated time: 15-20 min.

Quantitative Sensory Testing

Quantitative Sensory Testing (QST) is a way of assessing large and small sensory nerve fiber function using several different stimuli. The method has been verified using different protocols, and will be conducted in accordance with the original protocol by German Research Network on Neuropathic Pain (DFNS)[78]. All measurements are made on the dorsum of the foot. The protocol consists of seven tests measuring 13 parameters including, but in this study, we will only assess thermal detection. The tests will be performed using a thermal sensory testing device. Cold detection threshold (CDT) and warm detection threshold (WDT) are measured and calculated as a mean of three consecutive measurements. The thresholds are to be obtained with ramped stimuli increasing by 1 degree Celsius per second, terminated as the subject presses a button. Cut-off temperatures are set at 14 and 50 degrees Celsius, and the baseline temperature is set at 32 degrees Celsius, as this represents the mean value of skin temperature.

Estimated time: 10 min

Vagus Test Procedure

A commercially available handheld device (Vagus™, Medicus Engineering Aps, Aarhus N, Denmark) will be used to test the autonomic nervous system and hereby the autonomic neuropathy. The participant will hold the device during four steps, where the device will 1) measure the heart rate at rest, 2) measure the heart rate response from laying position to standing position, 3) measure the relationship between heart rate during expiration and inspiration, and 4) measure the heart rate conditions during exhalation with a resistance of 40mmHg and at rest. The latter will not be performed if the subject has known proliferative diabetic retinopathy or other known diseases with risk of retinal bleeding. The test is defined as abnormal if Cardiac Autonomic Neuropathy (CAN)-Score is ≥2.Estimated time: 15-20 min.

COMPASS-31

A questionnaire about symptoms of autonomic neuropathy (attached). Gives us the opportunity to measure non-invasive and stratify people with and without neuropathy. Will be filled on iPadby the participants, and entries are transferred to REDCap.

Estimated time: 10 min.

Michigan Neuropathy Screening Instrument (MNSI)

MNSI (attached) will be used as a part of the screening process for diabetic neuropathy. MNSI includes two separate assessments: a 15-item self-administered questionnaire (filled in REDCap by the participants) and a lower extremity examination that includes inspection and assessment of vibratory sensation and ankle reflexes. Estimated time: 15 min.

Assessment of muscle strength

Isometric handgrip strength will be assessed by a hand dynamometer (NC70144, Procare.dk, Denmark). Each subject is instructed to exert maximal contraction force with their dominant hand (defined as their "writing hand"), when standing in an upright position and the hand tested pointing downwards, parallel to the body, and unsupported. Each subject repeats the test three times. The maximal contraction level of the three trials is selected for further analysis.

Isometric measurement of knee extension strength will be measured with hand-held dynamometer. Similar procedures as for the arms.

Estimated time: 5 min.

Test for postural control

Subjects will be tested standing on a force platform (Plux Biosignals S.A, Arruda dos Vinhos, Portugal) during quiet bipedal stance in four different sensory conditions each of 35 seconds: i) eyes open, standing on a firm surface, ii) eyes closed on firm surface, iii) eyes open on soft surface, iv) eyes closed on soft surface. The trial including four sensory conditions will be recorded in one sequence and repeated two times separated by short breaks (30-60 s) in between. Subjects are asked to stand in the middle of the force platform as quiet as possible. All tests are performed barefoot, and the feet position is marked on the platform itself and on the soft foam pillow, during soft surface conditions. Soft foam pillow characteristics are: 48x40x6 cm (length, width, height), density 87 kg*m-3 (O'live Balance pad, Denmark). If subjects cannot hold their feet close together during the test, the distance between the widest spacing of the inner feet is measured and verified before all trials. Subjects are allowed a maximum of three attempts for each sensory condition. However, if they fail to complete the full 35 seconds of recordings in either of these attempts, this particular condition is skipped and not included in the analysis.

The vertical forces are extracted from the force platform (sampling rate at 1 kHz, Open Signals v. 1.2.8). The Center of Pressure (CoP) position versus time is extracted The Math Works, Inc., Natick, Massachusetts, United States of America. First and last 2.5 seconds are excluded after low pass filtering (15 Hz Butterworth, 2nd order and zero lag). The CoP range and velocity, Anterior-Posterior (AP) and Medial-Lateral (ML) directions, are calculated for each sensory condition. Average parameters from the three trials, for each of the four sensory conditions are used for analysis. The coefficient of variation for the four different conditions is extracted in a pilot study of seven healthy adults (aged 24-31years) and is <8.1% for both CoP range and mean velocity. Both CoP range and mean velocity are extracted in the 30s analysis window. The CoP range is extract subtracting the maximum value by the minimum CoP position and the mean velocity is calculated dividing the total CoP displacement by it respective time window (30s).

Estimated time: 10-15 min.

Assessment of gait function

The Timed up and Go (TUG) test measures time acquired to walk three meters back and forth as fast as possible although running is not allowed. The subject starts from a sitting position in an armchair (seat height approximately 43-47 cm) and asked to rise from the chair on an "three, two, one, go" start-signal and walk three meters, then turn around at the mark on the floor, and walk back to the chair and sit down again. Time is measured from the "go" signal and stopped when the subject again is positioned in the chair using the back

rest. All subjects are asked to wear supporting shoes and subjects not using such shoes are asked to perform the test barefooted.

The Chair Stand Test (CST) assesses lower body strength and power through the ability to rise from a chair and sit back down. Test subjects are required to stand up from a standard chair to a fully extended standing position with their arms folded across their chest. From the sitting position, the subject stands completely up, then completely back down, and this is repeated for 30 seconds. The total number of complete chair stands (up and down equals one stand) is counted.

Usual gait speed (UGS) takes place over 6 meters and composites of 4 meters of measuring, 1 meter of acceleration and 1 meter of deceleration. Subjects must walk at their own pace. Time <0,8 m/s (5 seconds on 4 meters), is viewed as cut off.

Estimated time: 5-10 min.

Assessments of OCT, Fundus and Visual Examination

All subjects will be tested with Optical Coherence Tomography (OCT), fundus photo and visual examination. Eyes will not be dripped. OCT is a non-invasive scan of the retina to measure the size and layer in combination with a fundus photo of the retina to assess the visual nerve and macula. If the subjects recently have been scanned at Aalborg University Hospital within 3 months, there is no need of a new one in case of stable eye-condition. The eye department at Aalborg University Hospital will assess the pictures.

Estimated time: 30 min.

5. DATA COLLECTION

Patient data will be collected using the EPJ. Physical data achieved doing the study will be stored in locked desks with locked doors. The door will be locked if the room is left empty. Computer equipment is borrowed by the North Jutland Region and is password protected in accordance with current guidelines. E-mails will only be sent through encrypted servers and mails, also in accordance with current guidelines.

6. DATA ANALYSIS

6.1. Sample size considerations

The total number of individuals that will be recruited for the study is based on previous cross-sectional or exploratory studies on diabetes, skeletal parameters, neuropathy, fall risk and postural control[45,46,79]. The primary concern was to assemble a study population large enough to permit multiple testing. Therefore, by applying a risk of 5% for type 1 errors (2α) and a risk of 20% for type 2 errors (β), we estimated that 300 people with T2D divided into the respective groups (F-/N-, F+ and N+) were acceptable for the procedures to avoid standard errors in analyses.

6.2. Statistical methodology

Mean and Standard deviation will be used as descriptive statistics for Gaussian distributed variables, median and 75 percentiles for skewed distributions. T-tests for two samples and multiple linear regression will be used for Gaussian distributed variables or variables who can be log-transformed. For non-Gaussian distributed variables non-parametric statistics will be used.

6.3. Statistical significance

We use a statistical significance level of $\alpha < 5$ %.

6.4. Missing data

Missing data, interpretation errors, coagulation defects are considered missing values and will not be included in the analysis. In this case, each sample requisition is numbered (N). Furthermore, samples that cannot be measured in numerical values are categorized as missing values.

6.5. Procedures in change

The procedures for change in original study plan are stated at publishing scientific material. Furthermore, the local scientific committee and GCP-units are notified as required of these changes.

7. DATA AND SAFETY MONITORING PLAN

7.1. Data

The project will be registered at the North Denmark Region Research Department according to the GDPR regulations. All sensitive data will be stored in either REDCap, on the protected server of Aalborg University Hospital or locally at DISK´s in DXA and HR-pQCT machines. Data will be stored anonymized after termination of the project.

7.2. Establishing of biobanks

Research biobank

Blood will be stored in a research biobank for later analysis. The blood is cooled down to -80 degrees and stored. Blood is handled by the research bio. Med. Lab. Technologists to safekeeping of EthyleneDiamineTetraAcetic acid (EDTA)-full blood, EDTA plasma, lithium-heparin plasma and serum. Subjects will be asked permission for storage in the research biobank.

Biobank for future research projects

Subjects will be asked permission to store an additional blood sample of 24 ml blood as well as an additional 8 ml urine sample from the 24-hour urine sample in a separate biobank with the perspectives of conducting future research projects. Only around 10 % of the tissue from skin biopsies is used for analysis. If the subject consents, excess material will be transferred back to Aalborg for storage in the biobank for future research. If the subject does not consent, excess material will be destroyed after analysis. Subjects will receive separate written information material about the additional blood sample, urine sample and storage of the excess skin tissue and will have to sign a separate consent form. Material will be stored in the separate biobank for 15 years after project termination or until a subject wants it destroyed. Use of material from the separate biobank will require permission from the regional ethics committee. Data will be handled in accordance with GDPR.

The collected and stored material will be encrypted after the project has been terminated via a serial number which cannot be related to the test subject. This number can however be related to the test subject's data and will be handled confidentially. If the test subject decides so, the material will be destructed after project termination. The project is covered by the North Jutland Region joint directory. The material will be stored with opportunity of identification in accordance with the North Jutland Region joint directory. This will be

linked to an identification number if the approval from the North Jutland Region joint directory expires before new ways of use have been tested.

The biobank will function as a database, where additional and potential important information can be collected. This database will be used in future studies. Reasons are:

- Some biomarkers are in such low concentration that it is uncertain to perform routine analysis. In time, technology will improve thus providing an opportunity for new and smarter ways of testing. Therefore, this is of great research potential that the samples are kept.
- Some biomarkers are not discovered and therefore not available doing the trial.

There are found no risks associated with storage of these samples in the biobank.

8. STUDY LIMITATIONS

Some limitations must be considered in this study. First, all participants may not be able to complete all test procedures due to individual restrictions. Further, it is voluntary if subjects want to participate in skin biopsies, which is a limitation. Both PTT and QST require corporation from the subjects and include subjective measures of pain. As the measured thresholds are not purely objective, psychosocial factors might influence test results.

9. ETHICAL CONSIDERATIONS

The trial will be conducted in compliance with *Harmonised Tripartite Guideline for Good Clinical Practice (*ICH GCP)[84] and applicable regulatory requirements, and in accordance with the Helsinki Declaration[85] for biomedical research involving test subjects. The methods used have been tested and performed in several studies both in Denmark and abroad and no long-term side effects have been reported. The risks associated with the project are few as low dose radiation is used and the tests imply limited risks. The potential benefits in terms of well-being are large and are estimated to outweigh the potential risks.

9.1. Informed consent

Subjects interested in participating in the study will receive written information about the project (*"Dine rettigheder som forsøgsperson i et sundhedsvidenskabelig forskningsprojekt"* produced by *Den National Videnskabsetiske Komité (NVK))* and a folder of the study ("*deltagerinformation"*)). If the subject is interested in participating in the project after reading the written information, they will be asked permission to access medical records for screening in accordance with the Danish law. Medical records will be assessed for information on comorbidities and diabetes complications, relevant diagnostic imaging, medications, and previous biochemistry to ensure the potential participant is suitable for inclusion. If suitable for inclusion after screening medical records, they will be invited for a personal meeting, where all details for the study will be outlined by the investigator or a delegated assistant. The subject will be informed about the opportunity to bring a lay representative in advance of the meeting. The meeting will take place in a closed room without any disturbances, e.g. phone calls. Subjects will be invited for an additional visit at our center to sign the informed consent (IC) form and further screening. After signing the IC, the subject will have blood samples taken for screening. Furthermore, we will assess status of neuropathy with questionnaires and physical examination including biothesiometry, NC-stat-DPNCheck, orthostatic blood pressure and Vagus

test, and the subjects will receive equipment and instruction for the 24-hour urine sample to collect before the study day.

The subjects must be fully informed of their rights and responsibilities while participating in the trial as well as possible disadvantages of potential risks. The investigator must ensure the subject has ample time to come to a decision whether to participate in the trial. A voluntary, signed, and personal dated IC must be obtained from the subject before any trial-related activity. The responsibility for seeking IC must remain with the investigator, but the task may be delegated by the investigator to a medically qualified person, in accordance with local requirements. The IC must be signed and personally dated by the person who seeks the IC before any trial-relate activity. Subjects will be informed that information given in relation to the project before signing the IC will be passed on to the investigator.
If information becomes available that may be relevant to the subjects willingness to continue participating in the trial, the investigator must inform the subject in a timely manner, and revised IC must be provided and a new IC must be obtained.
Participants have time for reflection and an additional informational meeting (with participating relatives etc.) will be scheduled.

The given information will include:

- That this a scientific project investigating the T2D bone phenotype and potential risk factors of diabetic bone disease including diabetic neuropathy.
- Information regarding the different tests involved including risks.
- That withdrawal from the project is possible at any time.
- That data collected will be published in an anonymized form, without any traces of personal data
- That data collected will be safely stored in accordance with the rules in force.
- The IC gives the investigator, the sponsor, and the sponsor's representatives as well as any supervisory authority direct access to obtain information in the patient's medical record, etc., including electronic medical records, in order to see information about the subject's health conditions, which is necessary as part of the implementation of the research project as well as for control purposes, including self-control, quality control and monitoring, which they are obliged to carry out.

9.2. Risks and side effects

As the project is mainly observational and contains no interventions the overall risk for the subject is extremely limited. No changes/adjustments in medication will be made and there will be no risk for hypo/hyperglycemia. The main issue for the participants is the fact that some time is needed for the conduction of the examinations. Furthermore, a small amount of transient pain might be felt during some of the examinations; however, no lasting side effects are reported regarding any of the examinations. To minimize the risk of side effects the examinations will be conducted by trained personnel, and all equipment will be sorely tested before usage on participants.
Different measurements may possibly reveal pathological conditions among subjects. The participants will be informed that the study might find additional medical conditions and they will be given the choice whether to be informed or not if this is the case. All findings, which are categorized "abnormal" or outside the normal range will be conferred with a medical doctor involved in the study, who will decide the plan and inform the

subject of this. This might lead to further examinations, which will be offered to the subject. If the subject has declined the opportunity to receive any information, this will be respected.

Listed below are the potential risks and side effects of the different examinations included in the protocol:

- **Blood and urine samples:** Almost negligible risk of infection, small bleeding, or pain around syringe insertion. 88 ml blood will be taken from each participant including screening samples. If subjects consent for samples for the biobank for future research projects, additionally 24 ml of blood will be taken, bringing the total amount of blood taken to 112 ml. If any blood samples come back with elevated or potentially dangerous results, the staff will act accordingly and discuss these findings with a medical doctor. No side-effect regarding urine samples.
- **DXA and HR-pQCT:** The risks of adverse reactions from the DXA or the HR-pQCT scans are minimal due to the low dose of radiation applied through the test. In theory this will increase the life-time risk of cancer from approximately 25% to less than 25,00002 %. No children or pregnant women are included in the project. The DXA scan may show sign of osteoporosis, which is an asymptomatically condition, mostly diagnosed after a fracture have occurred. Thus, it will be an advantage for subjects to have this diagnose assessed prior to any fracture occurrence. If osteoporosis is diagnosed, they will be referred to their general practitioner for further assessment and treatment.
- **Microindentation:** Participants will experience slight discomfort from microindentation due to localized pain in relation to the insertion site and, rarely, hematoma. Complications such as hematoma from microindentation are rare. There is a small risk of infection and reaction to local anesthesia such as redness, itching and subcutaneous hematomas.
- **PTT:** Participants might feel a small amount of transient pain during the examination. No lasting pain will occur.
- **Skin biopsy:** Participants might feel a small amount of transient pain during and a small bleeding. No lasting pain will occur.
- **OCT, Fundus Photography and visual examination:** It is a common procedure performed by trained personnel. Eyes will not be dripped.
- **Vital signs and body measurements, 12-lead ECG, skin AGE Reader, Vagus™, QST, NC-stat DPNCheck, force platform, gait function and muscle strength assessments:** No risks or side-effects.

9.3. Benefits to subjects

The study participants will not necessarily benefit directly from participation in the study. However, they will gain information about their general health including bone health, nerve function, balance and muscle function, body composition including fat and muscle mass and distribution. Individual participants may be diagnosed with illness(es) that may require treatment; in that case, they will be referred for relevant treatment.

Furthermore, the findings might help them later due to a better understanding and prevention/delay of the condition. In addition, the study might help their fellow diabetics, as this will emphasis the awareness of fractures as a complication to diabetes, which both subjects and the scientific community sorely needs.

9.4. Costs to subject

No r cost for the subjects is associated with this particular project.

9.5. Compensation to subjects

Transport expenses are covered according to current guidelines. No other economical compensation will be paid. In case of unforeseen side effects, the subjects will be compensated in accordance with the Danish Health Act and "*Patienterstatningsordningen"*.

9.6. Provision for vulnerable subjects

The participants are not expected to be any more vulnerable than the average patient is, and the trial does not contain any particularly distressing examinations.

10. ECONOMY

The initiative for this project has sorely been made by sponsor and main supervisor Peter Vestergaard and the primary investigator Julie Lindgård Nielsen.

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11. PLAN FOR DISSEMINATION OF FINDINGS

The study is planned to be submitted to internationally acknowledged scientific magazines. All data are published in anonymized form. The results will be published regardless of submission denial or whether the results are positive, negative, or inconclusive. Further dissemination might include national and international conferences and topical meetings. The subjects will receive information of the findings at the end of the study. The project will be prospectively registered to www.clinicaltrials.gov.

12. INSURANCE

The study is conducted at and covered by Aalborg University Hospitals insurance.

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14. APPENDICES

Compass-31

See separate document.

Michigan Neuropathy Screening Instrument (MNSI)

See separate document.

Flowchart of study inclusion