DK PRECISE

PREvention of **C**ancer by **I**ntervention with **SE**lenium

Biobank studies following an investigator-initiated randomised, double-blinded, one-centre clinical trial of selenium supplementation versus placebo in healthy elderly Danes

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Proposed biobank investigations and investigators

- **(I)** The effect of selenium supplementation on metabolic response – Results from a randomized controlled, double-blinded trial in an elderly Danish population
- **(II)** A randomized controlled double-blinded trial of selenium supplementation and risk of type-2 diabetes, as assessed by HbA1C, bilirubin and adiponectin concentrations
- **(III)** Large-scale analysis of Gene x Nutrient interactions on metabolic and CVD-related outcomes
- **(IV)** Effect of supplementation with high-selenium yeast on bone turnover markers - Results from a randomized, controlled, double-blind trial

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Introduction:

In this short study protocol we apply, as requested by the committee, for permission to analyse biological material in a biobank from the completed and previously approved DK PRECISE (Denmark PREvention of Cancer by Intervention with Selenium) trial (Journal ID: 19980186).

With the aim of showing the feasibility of conducting a large randomised controlled trial of selenium in cancer prevention in European populations of relatively low selenium status, both the UK and Denmark set up PRECISE pilot trials, recruiting elderly subjects from 1998 to 2001, using the same study protocol. In the UK PRECISE pilot trial, 501 healthy volunteers received six months of supplementation with 100, 200 or 300 µg/day selenium-enriched yeast or placebo-yeast. In UK sub-studies the effect of selenium supplementation *vs*. placebo has been investigated for mood, thyroid function, plasma lipid concentrations, and biomarkers of type 2 diabetes risk. Six months of selenium supplementation was found to have no effect on thyroid function (1). Meanwhile, modestly beneficial effects were found for plasma lipids (2) and no diabetogenic effects were found on the type 2 diabetes biomarker adiponectin (3). Previously, a study based on DK PRECISE biological material has investigated the effects of selenium on activity and gene expression of antioxidant and xenobiotic metabolising enzymes (4). Stored DK PRECISE serum and plasma samples have now been analysed for lipids, thyroid function and selenium concentrations. This facilitates a critical reappraisal of the results from the UK PRECISE pilot trial in a population of similar selenium status. A considerable benefit over the UK study is that the DK PRECISE trial continued for five years, enabling assessment of trend, and both short-term and long-term supplementation. The UK research group collaborate with us for these studies, with the first manuscript, for the lipids investigation, submitted to the Journal of Nutrition in November 2014. The reworking of the DK PRECISE trial has renewed interest for the trial, with new proposed investigations based on biological material collected during the trial. This protocol is the formal application to perform three such investigations.

Background

Selenium, an essential trace mineral, has a wide range of health effects when incorporated as selenocysteine into selenoproteins (5, 6). Early studies in areas of low selenium status have implicated selenium in cardiovascular disease (CVD) risk (7, 8). In Finland, increased cardiovascular morbidity and mortality were observed in men with low serum selenium (7) while selenium supplementation protected against cardiomyopathy in the Keshan province of China (8). Cardiovascular benefits of selenium could be mediated by the ability of selenoproteins, such as glutathione peroxidase and selenoprotein S, to combat the oxidative modification of lipids, inhibit platelet aggregation, and reduce inflammation (9-15). However the evidence that selenium status affects coronary heart disease risk is equivocal (16-20), with a recent Cochrane review flagging major gaps in the available trial evidence, especially with regard to long-term selenium supplementation trials (20).

The potential of the selenoproteins to protect against oxidative stress led to the expectation that selenium would be protective against type-2 diabetes, and indeed in the 1990s, selenium (as selenate) was shown to have anti-diabetic and insulin mimetic effects (21). However, more recently, findings from observational and experimental epidemiological studies have raised concern that high selenium exposure may lead to type-2 diabetes or insulin resistance, at least in well-nourished populations (22). A role for the essential nutrient selenium (Se) in bone health is increasingly recognized (23) as exemplified by the recent finding that bones are preferentially supplied under conditions of Se deficiency (24). *In vitro,* Se in high doses may prevent bone resorption through inactivation of osteoclasts (25). This is consistent with an observation in a human study among postmenopausal women, where Se status was inversely related to bone turnover (26). Effects of selenium supplementation on bone metabolism have never previously been assessed in human trials.

In the DK PRECISE trial the effect of selenium supplementation on plasma lipid concentrations and thyroid function has been evaluated (27, 28). Additional investigations based on biobank material, could further elaborate our understanding of the role of selenium intake in cardiometabolic and endocrinological health. One proposed investigation, aims to evaluate the metabolic response following selenium supplementation using a metabolomics approach (I), while another aims to investigate effects on type 2 diabetes biomarkers (II). A third investigation, benefiting from the trial design, will examine interactions between dietary factors

and genetic variants, that play an important role in contributing to the risk of developing obesity (III). A fourth investigation will assess the short-term and long-term effect of different doses of selenium supplementation on selected circulating bone formation (P1nP, osteocalcin, BAP) and resorption (CTX) markers.

Study design

Setting and participants

The DK PRECISE pilot study (ClinicalTrials.gov ID: NCT01819649) was a single-centre, non-stratified, randomized, double-blinded, placebo-controlled, multi-arm parallel clinical trial with four groups (allocation ratio 1:1:1:1). The sample size of this pilot study was set at 500 participants which was considered sufficient to draw reasonable conclusions about recruitment, adherence, and loss to follow-up while keeping the costs within reasonable bounds. No formal power calculations were performed. The funding necessary to conduct the international PRECISE trial was not secured and therefore it never took place.

Participants were males and females aged 60-74 years from the County of Funen, Denmark. Invitation letters were sent out based on a random sample from the Danish Civil Registration System. From November 1998 to June 1999, we invited 2897 potential participants of whom 630 accepted the invitation for a visit to Odense University Hospital where they were screened for inclusion. Exclusion criteria were: (i) a Southwest Oncology Group performance status score greater than 1; (ii) active liver or kidney disease (alanine-aminotransferase, alkaline phosphatase, bilirubin or urea two standard deviations above the normal reference range); (iii) previous diagnosis of cancer (excluding non-melanoma skin cancer); (iv) diagnosed HIV infection; (v) receiving immunosuppressive therapy; (vi) unable to understand written and spoken information; (vii) receiving ≥ 50 µg/d of selenium supplements in the previous 6 months (by patient report). Participants deemed suitable for inclusion provided blood samples and were given yeast tablets for an open-label 4-week placebo run-in phase. After this, potential participants returned for a second visit for a final evaluation of inclusion and exclusion criteria, participant adherence and satisfaction during the run-in phase. Good adherence was defined as taking more than 80% of the run-in phase tablets assessed by tablet count. The 491 subjects who met the inclusion criteria, displayed good adherence in the run-in phase and gave written informed consent, were enrolled and randomized to 0, 100, 200 or 300 µg of selenium daily. The regional Data Protection Agency and Scientific Ethical Committees of Vejle and Funen counties approved the study (Journal nr. 19980186).

Randomization and interventions

Randomization was computer-generated, blocked and non-stratified and was performed at the Division of Epidemiology & Biostatistics, University of Arizona, Arizona Cancer Center. A badge number system secured blinding and correct distribution of selenium doses. The responsibility of distributing tablets was placed with pharmacists at Odense University Hospital. Participating couples living at the same address were allocated to the same intervention.

The intervention agent was the selenium-enriched yeast SelenoPrecise© and tablets were formulated and packaged by Pharma Nord ApS, Vejle, Denmark. The placebo agent was an inactive spray-dried baker's yeast, comprising 250 mg of yeast placebo, 80 mg of cellulose, 65 mg of dicalcium phosphate, and ≤5 mg of other inactive ingredients, identical in appearance to the selenium tablets. Both intervention and placebo tablets were coated with titanium oxide in order to obtain identical smell and taste. Tablets were packaged in blister packs of 28 tablets, 7 x 4. Participants, research staff and investigators were blinded to treatment.

Sample and data collection and study outcomes

Participants were evaluated at Odense University Hospital at baseline and at 6, 12, 18, 24, 36 and 60 months. Demographic data, medical history including medication and supplement use, and food-frequency questionnaires were collected at baseline. During each visit, medical status was ascertained, side effects were registered and tablets were counted. Adherence was defined as in the run-in phase. New tablets were handed out except at the 60-month visit. Blood was drawn at 6, 12, 18, 24 and 60 months. Participants were non-fasting. Heparinised plasma, serum, whole blood, red blood cells and buffy coat were prepared and stored at – 80 °C. Reasons for participant withdrawal were recorded.

The pre-specified primary outcome of the pilot study was to determine recruitment, adherence and drop-out rate of the volunteers to ascertain the viability of conducting the main PRECISE trial in Denmark. The pre-specified secondary outcomes were: (i) to determine the number of staff necessary to conduct the main PRECISE study; and (ii) to perfect questionnaires and case report forms used at the participant trial visits.

Previous biochemical analyses

Total selenium at baseline and at the 6-month and 5-year visits was measured in lithium-heparin plasma at LGC Limited, Teddington, United Kingdom, by inductively coupled-plasma mass spectrometry (ICP-MS) with external calibration. The sample dilutions were introduced into the plasma *via* a micro-flow quartz concentric nebuliser, operating in pumping mode at 0.1 revolutions per minute (rpm), and a Scott double-pass spray chamber cooled to 2°C. The selenium isotopes ^{77}Se, ^{78}Se and ^{82}Se were measured in both H_2-mode and He-mode using collision cell ICP-MS (7700x, Agilent Technologies, UK) to reduce the interferences on the selenium isotopes. Each analysis comprised three replicate measurements. Germanium was added online as an internal standard to correct for any instrumental drift. Additionally, 2% methanol was mixed online in order to compensate for differences in carbon content between the samples and standards which may cause variances in ionization efficiency leading to erroneous results. Since the Se concentrations calculated for all the measured isotopes agreed well, only the data for ^{78}Se in H_2-mode was reported throughout. All reagents were of the highest purity. Methanol (Optigrade, LGC, UK) and nitric acid (UltraPure, Romil, UK) were used throughout. A stock solution of 1000 mg kg^{1} Se (Romil) was used to prepare freshly the working calibration standards (0-50 ng/g selenium) by gravimetric dilution in 0.5% (v/v) nitric acid. A matrix certified reference material, BCR-637 Human Serum, with a certified selenium concentration of 81 ± 7 µg/L selenium (density corrected 79.1 ng/g), was used for quality control of the total selenium measurements. The selenium concentration found for BCR-637 was 78.3 ± 2.7 µg/L Se (16 independent replicates) indicating good accuracy of the method. The intra-assay coefficients of variation (CVs) ranged from 0.5% for samples of high-Se concentration to 3% for samples of low-selenium concentration. The inter-assay CV was 3.4%.

Total and HDL cholesterol at baseline and at the 6-month and 5-year visits were measured in lithium-heparin plasma at the Department of Clinical Biochemistry, Odense University Hospital, Denmark, using an Architect c16000 analyzer (Abbott, Wiesbaden, Germany) with dedicated reagents. Measurements were performed by enzymatic colorimetric analysis. Traceability for total cholesterol and HDL cholesterol was ensured through participation in the National Reference System for cholesterol (NRS/CHOL) established by the Clinical and Laboratory Standards Institute (CLSI) with isotope dilution-mass spectrometry as reference method and reference material from National Institute of Standard and Technology (NIST). Intra-/inter-assay coefficients of variation were 0.6%/0.8% for total cholesterol and 1.0%/0.5% for HDL cholesterol. As evidence of

equivalence in the analytical performance of the cholesterol-oxidase assays in the UK and Denmark, a comparison of total cholesterol on 44 serum samples produced a limit of variation of 2%.

Serum was used for measurement of TSH, FT3, FT4 and thyroid peroxidase antibodies (TPO-Ab) at the Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Denmark, between September 2013 and March 2014. All measurements were serial to avoid interassay variation. TSH concentrations were measured with a chemiluminescence microparticle immunoassay using anti-TSH antibody-coated paramagnetic beads as first step and acridine-tagged anti-α TSH-conjugate for detection. The analytical limit of detection (LOD) is < 0.0025 mIU/L, but a functional sensitivity requires interserial CV% < 20, and the documented LOD thus is ≤ 0.01 mIU/L. Intra- and interassay variation coefficients were < 2%. The analysis was performed on an Architect i2000 (Abbott, Wiesbaden, Germany). The normal range was 0.3-4.0 mIU/L. FT3 and FT4 concentrations were analysed using time-resolved fluoroimmunoassays based on back-titration principle and using second-antibody separation. Both assays use specific mouse anti-human monoclonal antibodies and Europium served as a fluorescence enhancer. Analyses were performed on AutoDELFIA equipment (Wallac, Turku, Finland). LOD was 1.5 pmol/L (FT3) and 2 pmol/L (FT4). Intra- and interassay variation coefficients were <5% and <4% (FT3), and <2% and <5.4% (FT4), respectively. Normal ranges were 4.3-7.4 and 9.9-17.7 pmol/L for FT3 and FT4, respectively. TPO-Ab concentrations were analysed with a time-resolved fluoroimmunoassay. Recombinant human TPO was used to catch TPO-Ab in the sample and detection was performed with a Europium-labeled anti-human IgG directed towards TPO-Ab. Analysis was performed on AutoDELFIA equipment (Wallac, Turku, Finland) with an LOD of 1 mIU/L. Intra- and interassay variation coefficients were 3.4% and 6.4%, respectively, while the normal range was < 15 mIU/L.

Proposed investigations biochemical analyses

For the proposed investigation of metabolic response to selenium supplementation (I), metabolomics analyses will be performed by Henrik Frandsen, Senior Researcher. Fødevareinstituttet, Afdeling for Fødevarekemi, Mørkhøj Bygade 19, 2860 Søborg. Heparinized plasma samples will be fractioned into three: Phospholipids, lipids and polar. All fractions will be analyzed using ultra high performance liquid chromatography (UHPLC) linked to a mass spectrometer in full scan mode.

For the proposed investigation of type 2 diabetes biomarkers (II), lysed red blood cells will be analysed for HbA1C, heparinized plasma for adiponectin and serum for bilirubin concentrations. All analyses will be performed at Department of Nutritional Sciences, Faculty of Health and Medical Sciences
University of Surrey, Guildford GU2 7XH, United Kingdom.
For the Large-scale analysis of Gene x Nutrient interactions on metabolic and CVD-related outcomes (III), 5 ml whole blood samples will be used for DNA extraction and genotyping. Genetic analysis will be carried out by LGC Genomics, UK. This project will use a targeted candidate gene approach to study important genetic determinants for metabolic and cardiovascular disease outcomes. Several genome-wide association studies have identified variants for obesity, type 2 diabetes and hypertension; however it is still not clear whether these associations are modified by dietary factors. Hence, these genetic variants will be chosen based on the literature search, genotyped and examined for their interaction with dietary factors (macronutrients) on metabolic and cardiovascular disease outcomes. The findings from the PRECISE study will be meta-analysed with the results from other UK cohorts, if needed, in order to increase the statistical power to identify small effect sizes of the genetic variants. The genotyping will be done using (K Bioscience Competitive Allele-Specific Polymerase chain reaction) KASP genotyping methodology. The PCR-based KASP™ genotyping assay is a homogeneous, fluorescence (FRET) based assay that enables accurate bi-allelic discrimination of known Single Nucleotide Polymorphisms (SNPs). Unlike other PCR based genotyping assays, KASP requires no labeling of the target-specific primers / probes, giving it a clear cost advantage. Some of the samples will be re-genotyped to test for the accuracy of the genotyping.
For the proposed investigation of bone turnover (IV), serum and plasma will be analysed for osteocalcin, P1NP, BAP and CTX at Metabolic Bone Centre, Northern General Hospital, Herries Road, Sheffield, South Yorkshire, S5 7AU, England.

Additional analyses for proposed investigations

For the Large-scale analysis of Gene x Nutrient interactions on metabolic and CVD-related outcomes (III), dietary information, obtained from Food Frequency Questionnaires (FFQs) will be analysed at the Dept. of Food and Nutritional Sciences, University of Reading, UK.

Statistical analysis

For the comparison of randomized groups in projects (I), (II), and (IV), all trial participants will be assigned to their randomized treatment group, irrespective of compliance (intention-to-treat analysis). The effect of different doses of selenium supplementation on changes in biochemical outcomes after 6 months and 5 years will be estimated by using linear mixed models (29,30) with fixed effects for treatment groups, follow-up times, and treatment-by-time interactions, and allowing for random between-subject variations These models provide the mean changes from baseline to 6 months and 5 years for each treatment group, as well as the differences in mean changes for the three active treatment groups compared with placebo (treatment effects). We will evaluate treatment-effect modifications by sex, baseline age group (< or ≥ 65 years), and category of body mass index (< or ≥ 25 kg/m^2), baseline plasma selenium concentration (< or ≥ 90 ng/g), and baseline outcome concentrations by including all main terms and interactions between treatment group, time, and the corresponding covariate as fixed effects in the above mixed models. In sensitivity analyses, we excluded visits after participants received outcome relevant medications at baseline or during the intervention period.

In addition to the intention-to-treat analysis, we will evaluate the cross-sectional association between plasma selenium and outcome concentrations at baseline and the longitudinal associations between changes in plasma selenium and outcome concentrations after 6 months and 5 years, where relevant. We will use linear mixed models with random intercepts, random time slopes, and fixed slopes for baseline selenium levels and selenium changes at 6 months and 5 years to estimate the mean difference in baseline outcome levels per 50-ng/g increase in baseline selenium concentrations (cross-sectional association), as well as the mean outcome changes from baseline to 6 months and 5 years for each 50-ng/g change in the corresponding selenium concentrations (longitudinal associations). We will also categorize baseline selenium concentrations and selenium changes into quartiles in the above mixed model and compared mean baseline outcome concentrations across quartiles of baseline selenium and mean outcome changes after 6 months and 5 years across quartiles of selenium change. Cross-sectional and longitudinal associations will be adjusted for baseline age (continuous), sex, smoking status (never, former, or current), alcohol drinking (≤ 2, 3–10, or > 10 drinks/week), body mass index (continuous), and changes in outcome relevant medications over time.

For the Large-scale analysis of Gene x Nutrient interactions on metabolic and CVD-related outcomes in project (III), a goodness-of-fit chi-square test will be performed to confirm whether the genotype counts are in

Hardy-Weinberg equilibrium. Comparison of the means between the groups will be analyzed by Student *t* test or one-way ANOVA. The χ^2 test will be used to compare the proportions of genotypes or alleles. Linear regression analysis will be used to examine the association between the genetic variant and metabolic and cardiovascular disease outcomes. The interaction between the genetic variant and dietary components on outcome measures will be examined by including the interaction term in the mixed-effect models. All reported *P* values will be two-sided. All analyses will be performed by using either SPSS version 21 or STATA version 11 or later (StataCorp, College Station, Texas).

Publication plan

Attempts will be sought to publish all results, positive, neutral as well as negative, in peer-reviewed, indexed international journals. Authorship will be determined according to the guidelines from the International Committee of Medical Journal Editors.

Ethical considerations

The proposed PRECISE trial biobank investigations will only start after approval from the Regional Ethics Committee has been received. The original trial was conducted in compliance with the guidelines of the Declaration of Helsinki in its latest form and the ICH GCP guidelines. The original trial population was elderly at the time of inclusion in 1998-1999, and the experimental intervention is a dietary supplement, with no conclusive evidence for the potential benefits and harms, in the trial population. For these reasons, the investigator group hereby apply for exemption for re-obtaining informed consent from trial participants.
The Large-scale analysis of Gene x Nutrient interactions on metabolic and CVD-related outcomes does not extensive mapping of the humane genome. Rather, genotyping of selected gene variants using KASP technology, is a hypothesis driven approach, without risk of chance findings.

Conclusion

In conclusion, biological material from the DK PRECISE trial, offers a unique opportunity to learn more about the role of nutrient status in general and selenium status in particular, for cardio-metabolic and endocrinological health. In this protocol we have proposed three such further investigations.

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Appendices

Appendix 1 – Project description of Large-scale analysis of Gene x Nutrient interactions on metabolic and CVD-related outcomes.