Estudi PILCHARDUS

STUDY DESIGN

This is a multicentre, randomized, nutrition interventional study to evaluate whether a sardine diet can improve the metabolic control in patients with T2D. Three participating CIBERDEM clinic units from across different parts of Spain will participate in the study.

Sample size calculation of the present study has been made ensuring a power of 95% and aiming for a variation of at least 0.7 in the mean HbA1c between the nutrition interventional group and control group. Thus, using a standard deviation (SD) HbA1c estimate of 0.7 in accordance with the literature (54) at a 0.05 significance level and two-sided testing (STATA.11 software), 26 patients are needed per arm. Allowing for a 20% dropout rate, 32 patients per arm are sought for inclusion.

The patients will be recruited in Barcelona (Hospital Clínic and Hospital Vall d'Hebrón) and Málaga (Hospital Carlos Haya). The study will focus on recruiting T2D patients at diagnosis stage or been treated only with diet. After the recruitment, 64 T2D subjects will be assigned into sardine group (32 subjects) or into control group (32 subjects).

During 2 weeks, all participants will attend a dietetic education session once a week to ensure the understanding and future compliance of the nutritional intervention. The nutritional intervention will consist of maintaining the dietary habits of the subjects in the control group and of replacing part of the daily intake of protein by sardine protein in subjects within the intervention sardine group. During the study, there is no intention to reduce the body weight in any group.

After this period of dietetic education, the subjects will start the nutritional intervention, which will last 6 months. The expected duration of the study is approximately 10 months, including 3 months of enrolment period, 1 month of nutritional education and a follow-up period of 6 months.

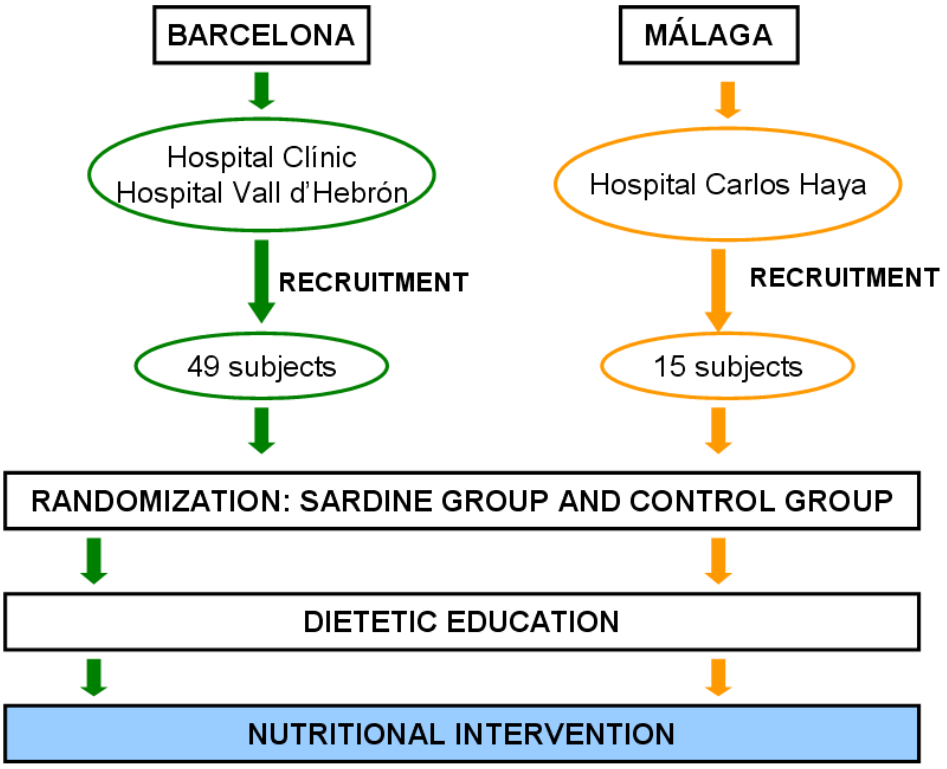

FIGURE 4. Scheme of study design

Inclusion criteria

1. Provision of informed written consent prior to any study-specific procedures
2. Age > 40 years
3. BMI ≥ 26 and < 35
4. Patients diagnosed with T2D at onset or treated only by diet
5. HbA1c between 6.5 and 8% (based on the last measured and documented laboratory measurement of the previous 3 months)
6. Usual consumption ≤ 3 fish per week

Exclusion criteria

1. Diagnosed hyperuricemia > 7mg/dl
2. Diagnosis of active neoplasic disease
3. Positive VIH or diagnosis of SIDA
4. Suffering from an acute illness which requires a recovery period higher than one week

5. There have been changes in the chronic medicacion in the previous 3 months before randomization
6. Known allergy or intolerance to fish or fish protein
7. Tryglicerides >500 mg/dl
8. LDL-cholesterol >250 mg/dl
9. Blood pressure readings higher than > 180/100 mmHg
10. Currently chronic treatment with oral steroids or non steroidal anti-inflammatory for more than 5 days, at least 1 month before randomization
11. Patients being treated with immunosuppressive drugs
12. Pregnant or breast-feeding patients
13. Serious acute vascular events (cardiac or cerebral) diagnosed previous 2 months before the randomization
14. Current or previous (within 6 months) participation in another study
15. Chronic renal insufficiency (creatinine >1,5 mg/dl)
16. Known liver function tests >2 times upper limit of normal
17. Any conditions that the investigator considers may render the patient unable to complete the study
18. T2D treated with antidiabetic oral drugs and/or insulin
19. Current or previous (within 3 months) intake of omega 3 suplements

During the interventional study, based on current standards of T2D care, doctors will introduce anti-diabetic oral drugs or insulin into the patient's treatment, where they consider it to be necessary.

Clinic measurements and procedures

- **Medical history**

During the first visit, medical history of each patient will be evaluated by a doctor and will consist on registering the following characteristics:

- sex, age, birthdate, birthplace (country), race
- Family history of diabetes, hypertension, dislypemia, cardiovascular disease or cancer
- Current suffering from hypertension, cardiovascular disease, dyslipidemia, any endocrinology disorder, other.

- History of type 2 diabetes: year of diagnosis, complications, treatment.
- Smoking habit

- **Physical examination**

During the first visit, at the third month of the intervention study and at the last visit, a physical examination will be done by a doctor and will consist of detecting any abnormal sign of each patient.

- **Medication checking**

Medication will be checked during the first visit, at the third month of the intervention study and at the last visit.

Suspected clinical adverse events (AE)

Clinical adverse events (AE) like undesirable signs and symptoms will be registered during the follow-up of the study.

- **Metabolomics analysis**

Metabolomics analysis of the blood samples will be performed by Metabolomic Platform of CIBERDEM in Tarragona.

- **Microbiota analysis**

The fecal bacteria composition will be determined in Hospital Clínic by real-time quantitative PCR (qPCR).

- **Anthropometric measurements**

Weight, standing height, arm, minimum hip, umbilical hip and waist circumferences will be measured in all participants following the International Standards for Anthropometric Assessment of ISAK (International Society for the Advancement of Kinanthropometry).

Body fat, lean mass and body fat distribution will be assessed by dual energy X-ray absorptiometry (DEXA) in subjects who will do the hyperinsulinemic euglycemic clamp

- **Dietary assessment**

Dietetic history

- Weight history: minimum weight, maximum weight, habitual weight in the last year, recent gain or loss of weight.
- Treatments for loss weight
- Number of meals/day and location
- Use of dietetic products (nutritional suplements,..)
- Food allergies and intolerances

Dietetic education

During 2 weeks before the start of nutritional intervention, in 2 sessions of 2 hours, dietetic education will be implemented by a clinical nutritionist in all the subjects, and will be designed to achieve that participants acquire the knowledge, skills, attitudes, and behaviour changes necessary to follow the nutritional prescription.

In the sardine group, the sessions will be focused on explaining the principles of the sardine diet, the health benefits associated to this food pattern, meals planning, food selection, food preparation and portion control. In the control group the sessions will be focused on explaining the principles of a balanced diet and maintaining their habitual food pattern or improving it if required. The dietary modifications will always be adapted to the participant's food preferences. The dietetic education will continue in the follow-up period, once a month; in individual sessions with a nutritionist, in order to reinforce the adherence to the nutritional intervention.

Dietary intervention

Subjects will be asked to complete a 4-day food diary (3 weekdays and 1 holiday) before the onset of the study to determine their usual energy intake. After review of the initial diet records, within each of the two study arms, subjects will be asked to include a fixed amount of sardine in daily meals (equivalent to a 30% of meat protein intake) as part of their usual diet (sardine group) or to continue on their usual diet alone (control group).

The proportions of macronutrient intake in the diet will not be modified, because we focus on changing the source of protein and fat of the diet, not on changing the usual dietary pattern or energy intake. In our nutritional intervention, we replace much of the

animal protein intake for sardine protein. Therefore, we are achieving an increase in fish protein and ω-3 fatty-acids only with the inclusion of one food in the diet, the sardine. In order to improve the nutritional adherence we will offer to the subjects a large variety of sardine foods (some of which have been developed specially for the study). Maintenance of usual lifestyle will be encouraged during the 24 weeks of the intervention period.

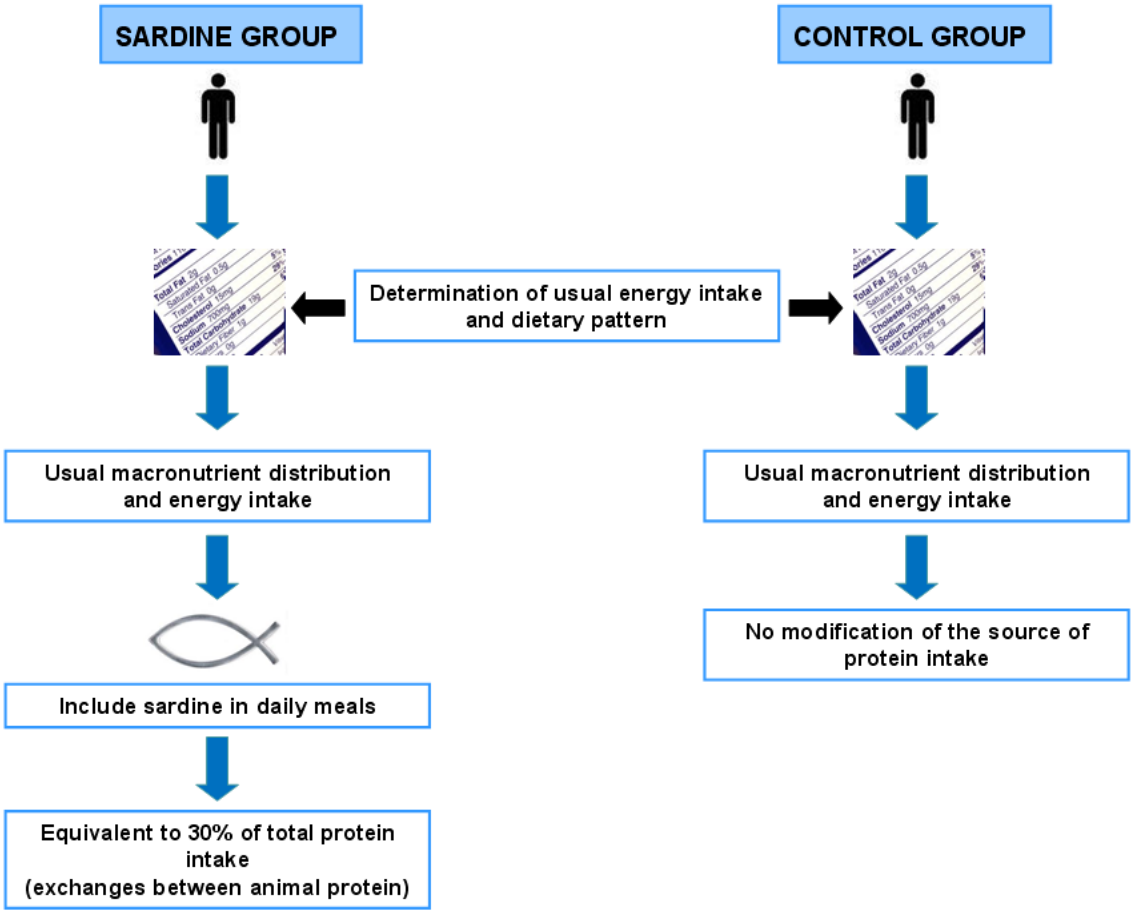

Figure 6. Design of dietary intervention

Sardine foods

Alicia Foundation is responsible for formulating different sardine foods from canned and fresh sardine to offer to the subjects a large variety of gastronomic use of sardine and to avoid monotonous daily sardine menus. Canned sardine will be supplied to the subjects during all the interventional period free of cost.

Alicia Foundation will develop different sardine based recipes containing 50 and 100g of canned oil olive sardines in order to be easily adaptable to the daily intake scheduled for each patient, and to facilitate patient adherence to the treatment. Moreover, Alicia Foundation will design a variety of meals including fresh sardine for increasing the variety of sardine preparations.

Diet compliance

Dietary intake will be monitored by the same dietitians throughout the study, with completion of 4-day diet record (3 weekdays and 1 weekend day) and food-frequency questionnaire (FFQ). Urine excretion of taurine and serum levels of ω-3 fatty-acids will serve to a biomarker of sardine consumption in subjects.

WORK PLAN

Screening Visit

All potentially eligible patients will undergo a combined screening/enrolment/randomisation visit. If the patient has a clinically stable HbA1c value performed within the prior 3 months, this may be used to determine eligibility. If it has not been measured within 3 months, it should be determined as part of the screening assessments in order to determine eligibility. If a patient is eligible, he/she will be informed of the study and will have to accept participating in it by signing the written consent.

Subjects will be given written and oral instructions on how to keep diet records by a nutritionist. Dietetic education will be done to all the patients during the next 4 weeks. In order to determine the usual energy intake and dietary pattern of the patients, participants will need to complete a 4-day diet record (3 weekdays and 1 holiday) which they will bring completed in the next visit (basal visit). Moreover, a food-frequency

questionnaire (FFQ) will be done during the visit. Finally, in the end of the screening visit, patients will receive the sterile material for collect urine and faecal samples that they must give back in the next visit (Basal visit).

Basal visit

Demography, medical history, dietetic history and concomitant medication will be recorded. A physical examination, anthropometric assessment and blood sampling for laboratory assessments will be taken. Urine and fecal samples collected by the patients will be stored in Biobank of CEK (Centre Esther Koplowitz).

Euro Qol and FFQ will be done. During the visit, the nutritionist will check the 4-day diet record of the patient and the correct understanding and use of the dietary education that has been done. Another visit will be planned specific for doing the hyperinsulinemic euglycemic clamp in a sub-cohort of 20 patients (10 from each group). Body fat distribution and composition by dual energy X-ray absorptiometry (DEXA) will be performed to those who will do the clamp.

Visits 1, 2, 4 y 5

The suspected clinical adverse events (AEs) will be recorded. If required, a targeted physical examination will be done. The specific adherence to the diets will be considered and FFQ will be done. The nutritionist will check the correct understanding and use of the dietary education by the patients. Moreover, the nutritionist will encourage their adherence to the diet and will evaluate any specific difficulties related to the interventional diet. In the visit 2 the patients will receive the sterile material for collect urine samples that they must to give in the visit 3.

Visit 3

The data clinic questionnaire and suspected clinical adverse events (AEs) will be recorded. A physical examination, anthropometric assessment and blood sampling for laboratory assessments will be taken. Urine samples collected by the patients will be stored in Biobank of CEK (Centre Esther Koplowitz). Concomitant medication will be recorded. The specific adherence to the diets will be considered and FFQ will be done. The nutritionist will check the correct understanding and use of the dietary education by

the patients. Moreover, the nutritionist will encourage their adherence to the diet and will evaluate any specific difficulties related to the interventional diet.

Visit 6: End of study and closing visit

The data clinic questionnaire and suspected clinical adverse events (AEs) will be recorded. A physical examination, anthropometric assessment and blood sampling for laboratory assessments will be taken. Urine and fecal samples collected by the patients will be stored in Biobank of CEK (Centre Esther Koplowitz). Concomitant medication will be recorded.

Euro Qol and FFQ will be done. During the visit, the nutritionist will check the 4-day diet recorded by the patient. Another visit will be planned specific for doing the hyperinsulinemic euglycemic clamp in the same 20 patients sub-cohort. Body fat distribution and composition by dual energy X-ray absorptiometry (DEXA) will be performed to those who will do the clamp. For patients who prematurely discontinued the study, only a value of HbA1c and AEs will be recorded at the closing visit. No further assessments are needed. The same is applicable for the earlier visits following a premature end.

Weekly telephone check-up

The patients will be contacted every week between their regular site visits by the investigator/study site personnel, to encourage their adherence to the diet, to evaluate any specific difficulties related to the interventional diet, to remind them of the next site visit and to detect any adverse events. An unscheduled visit may be conducted as a result of the phone interview.

- ***Data management and statistical analyses***

Data will be registered using written questionnaires from each patient. Lately, there will be quality checking in order to ensure correct introduction into the computerized dataset. Statistical analyses will be performed using STATA.11 software. The significance level is set up at $p < 0.05$ (two sided). No interim analyses are planned. Results will be expressed as means and SD if continuous variables or as % if categorical variables. ANOVA or Chi squared tests will be performed. Potential interactions of the diets with sex, body weight change and insulin sensitivity at baseline

on the measured variables will be tested by individual entry of terms and interactions terms into the ANOVA model. All data will be analyzed on an intention-to- treat principle.