Title: O-LIVE: Impact of Portuguese Olive Oil on Biomarkers in Healthy Volunteers

Project nº 171-CES/UCP

Porto, September 2022

Protocol

Title: O-LIVE: Impact of Portuguese Olive Oil on Biomarkers in Healthy Volunteers

Introduction

This research project, approved and funded, is part of a larger project titled "HSoil4Food - Healthy Soil for Food" which encompasses various work packages, one of which is a clinical research project now submitted to this Ethics Committee, titled "O-LIVE: Impact of Portuguese Olive Oil on Biomarkers in Healthy Volunteers".

Olive oil is a millennia-old product that is crucial for social cohesion and local economies in the Mediterranean region, particularly in Portugal [1]. Among the numerous food items, olive oil stands out as one of the most extensively studied in terms of its health benefits and contribution to the prevention of non-communicable chronic diseases [2]. There is ample evidence of the protective effect of olive oil against cardiovascular diseases, diabetes, oncological diseases, and neurological degeneration, the latter being responsible for 8 out of 10 deaths in the Western world [3, 4]. Olive oil has been associated with the reduction of obesity, body weight, body mass index, and waist circumference, which is a reasonable indicator of visceral adiposity [5]. It has also been shown to alter serum lipid profile, decreasing LDL cholesterol and increasing HDL cholesterol [6]. In addition, olive oil has been shown to increase oxidation rate, inhibit lipogenesis, and stimulate lipolysis, as well as affect the expression of receptors involved in weight regulation and fat utilization [7].

The health benefits of olive oil derive from its specific nutritional composition, including monounsaturated fatty acids, polyphenols, and phenolic compounds, which act on metabolism, modulation of inflammatory responses, and gene expression [8]. However, the composition of olive oil can vary considerably depending on the type of olive trees cultivation, soil management practices, as well as extraction and technological processes involved in olive oil production [9]. In the scope of a previous project ("Bio-n2-value - Biological tools to add and defend value in the main agri-food chains", NORTE-01-0145-FEDER-000030), it was demonstrated that similar monovarietal olive oils from different locations in the Trás-os-Montes region revealed different lipid and polyphenolic compositions [10, 11]. These significant differences in olive oil composition may explain the differences found in clinical results.

In this study, we will use an olive oil rich in polyphenols (locally and sustainably produced) and evaluate its effect on various important clinical parameters involved in chronic diseases. We hope that the results of this study will help support the relationship between consumption of olive oil with higher polyphenol content and overall health improvement.

Objectives

The main objective of this study is to evaluate the impact of ingestion of polyphenol-rich olive oil on important clinical parameters, such as anthropometric measurements, blood and urinary biomarkers, gene expression, and gut microbiota composition. To achieve this objective, a quasi-experimental clinical study will be conducted with the following tasks:

1) Recruitment of participants;

2) Intervention;

3) Periodic evaluation of the impact of olive oil ingestion on clinical parameters of participants, through:

3.1) Administration of questionnaires;

3.2) Anthropometric measurements;

3.3) Analysis of blood biomarkers;

3.4) Analysis of urinary biomarkers;

3.5) Analysis of gene expression;

3.6) Analysis of gut microbiota.

Methodology

This project will be carried out at the Center for Biotechnology and Fine Chemistry (CBQF) and at the School of Biotechnology of the Catholic University of Portugal, Porto campus.

<u>Task 1 - Recruitment of Participants</u>

This project consists of a quasi-experimental clinical study, whose target population is healthy adult volunteers. Participants will be recruited among men and women, aged 18 years or older, who are healthy and belong to the community of the Catholic University

of Portugal (Porto) and do not meet any of the exclusion criteria (described in the questionnaire). Individuals will be invited to participate voluntarily in this study, receiving detailed information about the research to allow for an informed and free decision. Individuals who agree to participate will sign an informed consent.

Task 2 - Intervention

Participants will include in their usual diet the daily intake of 30 mL of polyphenol-rich olive oil (intervention group) for 3 months. At the beginning (day 1), midpoint (between days 40 and 50), and end of the intervention period (day 90), participants will be assessed. The olive oil will be placed in dark-colored containers to minimize loss of phenolic content due to sunlight. The containers with the olive oil will be provided free of charge to participants at the beginning of each intervention period. Participants' adherence to olive oil intake will be verified through two processes: 1) by returning the containers at the end of each intervention period for the measurement and recording of the daily amount of unconsumed olive oil; 2) by quantifying hydroxytyrosol by GC-MS or LC-MS in 24-hour urine samples collected from participants who agree to this urine collection.

Task 3 - Periodic evaluation of the impact of olive oil intake

To investigate the impact of olive oil intake, participants will be evaluated at three established time points: at the beginning (day 1), midpoint (between days 40 and 50), and end of the intervention period (day 90). This evaluation will be performed by healthcare professionals and will consist of administering a standardized and validated questionnaire in person to obtain information on olive oil intake, dietary habits, and physical activity level.

Task 3.1 - Administration of Questionnaires

A standardized and validated questionnaire will be administered in person to participants to obtain information on olive oil intake, dietary habits, and physical activity level.

Task 3.2 - Anthropometric Measurements

Anthropometric measurements, obtained according to standardized protocols, include the measurement of height, body weight, waist circumference (WC), and collection of body composition data. Height and weight will be measured with a stadiometer and a scale, respectively, with participants barefoot. Waist circumference will be measured at the midpoint between the last rib and the iliac crest using a flexible measuring tape. Body composition data, including fat mass and lean body mass, will be collected through

bioelectrical impedance analysis (BIA). Body mass index (BMI) will also be calculated by dividing weight by height squared. In addition to anthropometric measurements systolic and diastolic blood pressure will be measured using a sphygmomanometer after the participant has rested for 5 minutes.

Task 3.3 - Analysis of Blood Biomarkers

This task aims to assess the impact of olive oil consumption on various blood biomarkers through the analysis of capillary blood samples. Capillary blood will be collected from a finger of the hand using a single-use lancet at three timepoints during the intervention study. The capillary blood samples (volume in µL) will be analyzed using the cobas® b101 device (Roche), a Point of Care equipment that allows for rapid quantitative measurement of glycated hemoglobin (HbA1c), estimated average glucose (eAG), triglycerides (TG), total cholesterol (CHOL), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), atherogenic index (CHOL/HDL), and C-reactive protein. These data will be used to calculate the visceral adiposity index (VAI) using the formulas described below:

- Male VAI : $\left[\frac{WC\ (cm)}{\left\{39.68+(1.88x\mathrm{BMI}\left(\frac{\mathrm{kg}}{\mathrm{m}^2}\right))\right\}}\right] \mathrm{x} \left[\frac{\mathrm{TG(mmol/l)}}{1.03}\right] \mathrm{x} \left[\frac{1.31}{HDL(\mathrm{mmol/l})}\right]$
- Female VAI : $\left[\frac{WC\ (cm)}{\left\{36.58+(1.89x\mathrm{BMI}\left(\frac{\mathrm{kg}}{\mathrm{m}^2}\right))\right\}}\right] \mathrm{x} \left[\frac{\mathrm{TG(mmol/l)}}{0.81}\right] \mathrm{x} \left[\frac{1.52}{\mathrm{HDL(mmol/l)}}\right]$

VAI: Visceral Adipose Index, WC: Waist Circumference, BMI: Body Mass Index, TG: Triglyceride, HDL: High Density Lipoprotein

Task 3.4 - Analysis of Urinary Biomarkers

This task aims to assess the impact of olive oil consumption on the urinary concentration of 8-hydroxy-2'-deoxyguanosine (8OHdG), a biomarker of oxidative DNA damage. Urine will be collected in a 100 mL container, and the concentration of 8OHdG will be quantified using an enzyme-linked immunosorbent assay (ELISA) method with commercially available kits. In addition, the concentration of creatinine in the corresponding urine samples will be determined using a colorimetric kit to normalize the urinary concentration of 8OHdG.

Task 3.5 - Analysis of Gene Expression

A buccal smear sample will be collected for subsequent DNA extraction and real-time quantitative PCR (Polymerase Chain Reaction) to determine the expression of selected genes, such as SOD1, CCL2, and NFkB, involved in inflammation and oxidative stress.

Task 3.6 - Analysis of Intestinal Microbiota

This task aims to evaluate the impact of olive oil intake on the composition of the intestinal microbiota through the analysis of fecal samples. Bacterial populations in fecal cultures will be analyzed by extracting microbial DNA and applying real-time quantitative PCR using specific primers based on 16S rRNA gene sequences. The impact on the metabolic activity of the intestinal microbiota will be evaluated in fecal supernatants in terms of: a) quantification of short-chain fatty acids and lactic acid by HPLC-UV, according to Gullon et al. [13]; b) quantitative analysis of the disappearance of important bioactive compounds previously identified in olive oil and the corresponding formation of metabolites using GC-MS/GC-FID.

Future Perspectives

With this project, we expect to identify whether olive oil with this lipid profile, phenolic content, and antioxidant properties modifies and impacts important serum biomarkers, including lipid and anti-inflammatory profiles, blood pressure, as well as anthropometric variables and intestinal microbiota composition. These results will help clarify the nature of the relationship between polyphenol-rich olive oil and health indicators. The definition of an effective dose of olive oil for shaping certain biomarkers and health indicators based on its polyphenol composition may be of great importance in nutritional therapy and may constitute an alternative approach for prevention and management of different chronic diseases. The results will be disseminated to various audiences, ranging from the academic community (scientific journal manuscripts), healthcare professionals (nutritional counseling), to the general public (mainstream media).

References

[1] Casado---Montilla, J., Carrillo---Hidalgo, I. (2019). Introducing olive---oil tourism as a special interest tourism. Heliyon 5 (12), e02975.

[2] WHO. (2016). Assessing national capacity for the prevention and control of noncommunicable diseases: report of the 2015 global survey. ISBN 9789241565363.

[3] Gavahian, M., Khaneghah, A., Lorenzo, J., Munekata, P., Garcia---Mantrana, I., Collado, M., Melendez---Martínez, A., Barba, F. (2019). Health benefits of olive oil and its components: Impacts on gut microbiota antioxidant activities, and prevention of noncommunicable diseases. Trends in Food Science & Technology 88, 220---227.

[4] Alkhatibab, A. (2020). Chapter 33 – Olive oil nutraceuticals and chronic disease prevention: More than an offshoot of the Mediterranean diet. The Mediterranean Diet (Second Edition). An Evidence---based Approach. Pages 363---370.
[5] Perona, J., Vogler, O., Sanchez---Dominguez, J., Montero, E., Escriba, P., Ruiz---Gutierrez, V. (2007). Consumption of Virgin Olive Oil Influences Membrane Lipid composition and Regulates Intracellular Signaling in Elderly Adults With Type 2 Diabetes Mellitus. Journal of Gerontology 62A, 256–263.
[6] Alkhattaf, N., Alraddadi, A., Aljarbou, M., Arnauti, M., Hammouda, S. (2020). Determining the correlation between olive oil consumption, BMI, and waist circumference in the adult Saudi population. Journal of Taibah University Medical Sciences 15---5:380---86.
[7] Forouhi, N., Krauss, R., Taubesm, G., Willett, W. (2018). Dietary fat and cardiometabolic health: evidence, controversies, and consensus for guidance, British Medical Journal 361: k2139.
[8] Visioli, F., Franco, M., Luchsinger, E., Willett, W., Hu, F., & Martinez-Gonzalez, M. (2018). Olive oil and prevention of chronic diseases: Summary of an International conference. Nutrition, Metabolism and Cardiovascular Diseases, 28(7), 649-656.
[9] Castillo-Luna, A., Criado-Navarro, I., Ledesma-Escobar, C., Lopez-Bascón, M., Priego-Capote, F. (2021). The decrease in the health benefits of extra virgin olive oil during storage is conditioned by the initial phenolic profile. Food Chemistry, 336, 127730.

[10] Rodriguez-Alcalá, L.M., Pimentel, L.L., Figueredo, I., Pereira, J.A., Gomes, A.M., Pintado, M. (2020). Compositional characterization of multi and monovarietal oils obtained from Tras-os-Montes region. Food Chemistry (em submissão).

[11] Rodriguez-Alcalá, L.M., Machado, M., Pimentel, L.L., Figueredo, I., Pintado, M., Gomes, A.M. (2020). Changes in the lipid and polyphenol composition of olive drupes as affected by oil elaboration. (em elaboração).

[12] Gullon, P., Gullon, B., Cardelle-Cobas, A., Alonso, J.L., Pintado, M., Gomes, A.M. (2014). Effects of hemicellulose-derived saccharides on behavior of lactobacilli under simulated gastrointestinal conditions. Food Research International, 64, 880-888.

Statistical Analysis Plan (SAP)

Baseline characteristics of participants were compared according to adherence to the Mediterranean diet. Means (SD) or percentages for each variable were calculated and the statistical significance of the differences will be assessed by one-way ANOVA and chi-squared tests.
All data will be assessed and compared using 95% confidence intervals (CI). Pearson

correlation coefficients (r) between each parameter will be assessed. If the Pearson coefficient is sensitive to data distribution, Spearman rank correlations can also be accounted.

Multivariable linear regression modelling can be used to compare adjusted differences between the different parameters. Finally, multivariable logistic regression will be used to estimate the association between the clinical and anthropometric parameters. A p value <0.05 will be considered as statistically significant.

Informed Consent Form (ICF)

Consent informed, clarified and free for clinical investigation

Note: This document is made in duplicate: the original remains in the process and the copy is obligatorily delivered to the consenting person.

Consent informed, clarified and free for clinical investigation considering the “Declaration in Helsinki” from the World Medical Association world (Helsinki 1964; Tokyo 1975; Venice 1983; Hong Kong1989; somerset west 1996; Edinburgh 2000; washington 2002; Tokyo 2004; Seoul 2008; Strength 2013)

O-LIVE: Impact of Portuguese olive oil in biomarkers of healthy volunteers

Information to the participant

This financed project is approved and included on a bigger scale project, entitled HSoil4Food “Healthy soil for food” that comprises other work packages, one of which a clinical research project entitled “O-live: Portuguese olive oil impact on biomarkers of healthy volunteers”, now submitted to this ethics committee. This project aims to assess the impact of the ingestion of a polyphenol rich olive oil in anthropometric parameters, serum biomarkers and gut microbiota of healthy volunteers. For this purpose, a quasi-clinical trial will be conducted, to which healthy adult volunteers will be recruited. At the beginning, middle and final of the study period, data regarding their food habits, anthropometric data (height, weight, waist perimeter, body composition by bioelectrical impedance), clinical data (blood pressure), finger prick to withdraw a drop of blood using the “point of care” technology to determine the participants lipid profile (cholesterol, triglycerides, glycosylated haemoglobin and C reactive protein), stool sample for the gut microbiota analysis and saliva sample for the gene expression in inflammatory and oxidative stress processes analysis. All the clinical data will be collected by a health care

professional from the UCP investigation team. The participants of the study will include in their diet an extra of 30 ml/day of extra virgin olive oil provided freely by the investigation team for 100 days. The present study was approved by the UCP Ethics Committee.

Olive oil is a food item very common in the Portuguese cuisine, being the olive oil provided for this study commercialized having the quality control, providing no expectable harm to the health of the participants. With this study, the participant will have the opportunity to have free olive oil for 100 days to include, raw, in their daily food routine while also having access to all the clinical results. The participation in this study is volunteered and the participant can drop out of the study at any point, asking for the destruction of all their clinical data. All the questionnaires' answers will be analysed within confidentiality and the anonymization will be maintained as the information is broadcasted. Data obtained could be used to clinical purposes presentations and scientific publications. There's no conflict of interests between the investigators and participants.

Privacy policies and data treatment

As a data subject, you may exercise the following rights at any time and as long as they are not deleted from the database, subject to the legal conditions necessary for their exercise: right of access, right of rectification, right of erasure, right of restriction of processing, right of data portability, and right of objection to processing. For further clarification on these rights, you may consult the information at this link: https://www.ucp.pt/en/direitos-do-titular-dos-dados, and use the following contact:

O Encarregado de Proteção de Dados (Data Protection Officer) da UCP

Nome: Dra. Frederica Campos de Carvalho

Contactos: +351 217214179

Email: compliance.rgpd@ucp.pt

declare that:

I have received the Participant Information text and have understood the explanation given to me about the objectives, methods, anticipated benefits, potential risks, and any discomfort related to the study that is intended to be conducted.

I had the opportunity to ask any necessary questions, and I obtained satisfactory answers, so that my doubts were clarified.

I have been informed that I have the right to freely refuse or withdraw my participation in the study at any time, without the need for justification, and to request the immediate deletion of all my data collected in the context of the study, without any prejudice or penalty for this fact.

I have been guaranteed that all data related to the identification of Participants in this study are confidential and that anonymity will be maintained, and they cannot be used for other studies.

I am not participating in any other research project at this time.

I am of legal age and have been informed about the objectives of the study, which will consist of voluntarily undergoing scheduled interventions, responding to questionnaires, anthropometric measurements, and collection of biological samples (blood, urine, oral swab, and feces), with confidential treatment of the data. I have been informed that I can withdraw from filling out the questionnaires at any time.

In accordance with the General Data Protection Regulation (GDPR), my contact data will be included in a file with the sole purpose of allowing communication with the Universidade Católica Portuguesa within the scope of this study.

I have been informed of my rights of access, rectification, and cancellation in relation to my personal data as provided by law.

Therefore, I willingly and freely agree to participate in the aforementioned study, I agree to undergo the proposed surveys and methods, and authorize the dissemination of the obtained results in the scientific community, with anonymity guaranteed.

The participant:

Date: Signature:

Main investigator:

Name: Prof. Doutora Marta Correia

Contact: mmcorreia@ucp.pt

Date: Signature: