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Original Article

Atherosclerosis experimental model and the effects of extra virgin olive oil on the heart and peripheral arteries of rats

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ABSTRACT

Atherosclerosis is a multifactorial disease resulting from the development and progression of atheromatous plaque. Thus, the nature and amount of lipids in the diet may act as a risk or protective factor for cardiovascular impairment. The extra virgin olive oil, due to its antioxidant power, was used in this study to evaluate its therapeutic effects on the atherosclerosis process. Male Wistar rats were divided into 6 groups: negative control, normocaloric diet and olive oil, positive control, high fat diet and olive oil, high fat diet and olive oil with fasting and dyslipidemia induction group with high fat diet/treatment with olive oil. After 90 days, blood was collected. Euthanasia was performed and carotid arteries, abdominal aorta and iliac vessels were removed for histopathological analysis. Areas of vacuolar degeneration and pyknotic nuclei were not found. Aorta histological analysis showed grade II lesions in all animals that consumed olive oil as treatment after induction of dyslipidemia, whereas only 20% of the animals in the group in which normocaloric diet and olive oil were administered throughout the experiment presented the same lesion degree (intimal thickening and presence of foam cells). The triglycerides/HDL index was significantly lower in the normocaloric diet group than in the dyslipidemia and subsequent ingestion of olive oil group ($p = 0.0159$). The continuous administration of a balanced diet and extra virgin olive oil has a potential positive effect on the lipid profile and subsequent formation of atheroma plaque.

KEYWORDS: olive oil, Mediterranean diet, atherosclerosis.

1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the world and represents a major public health burden [1]. Most cases are associated with atherosclerosis (AS), a chronic multifactorial inflammatory process [2]. AS affects mainly the artery intima layer, and this process begins through the oxidative species activity in endothelial arteries [3].

Low-density lipoprotein cholesterol (LDL-c) is mainly responsible for oxidation [4]; it takes part in the formation of atherosclerotic plaques [5]. Atheroma plaques encompass focal accumulation of lipids, fibrous proteins and inflammatory cells in the artery intima [6], leading to the formation of foam cells, followed by fatty degeneration of large arteries due to increase in smooth muscle cells, resulting in vessel occlusion [7] and thrombus formation following plaque rupture [8].

Epidemiological studies indicate that dyslipidemia is the major risk factor for CVD [9], and this may be due to several factors, including a deficient diet

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containing saturated fat. An example of this kind of diet is a highly palatable and caloric-rich diet called “cafeteria diet” or “occidental diet”, very often adhered by the current society [10].

Studies using an experimental model with rats showed that “Cafeteria diet” was associated with cardiac damage such as coronary perivascular fibrosis, in addition to higher insulin levels and serum cholesterol levels when compared to other diets. [11]. Therefore, a significant reduction in lipids and sugar intake is related to a reduced risk of coronary events [12].

Several studies have found that nutrients and food components could positively impact the lipid profile: monounsaturated and polyunsaturated fatty acids, soluble fiber, phytosterols, and polyphenols [13]. A Mediterranean diet component, the extra virgin olive oil (EVOO) is an important food with a phenolic profile that contains unique biological properties [14]. The Mediterranean diet has an inverse relation with atherosclerotic disease and it is associated with a lower incidence of ischemic heart disease [15].

Extra virgin olive oil polyphenols are responsible for decreasing LDL oxidation and increasing high-density lipoprotein cholesterol (HDL-c) in plasma. In addition, it contains a high content of monounsaturated fatty acids, a lipid profile that provides protection against oxidative damage, decreasing cardiovascular risk [14].

Thus, the aim of this study was to evaluate the biochemical and histopathological parameters for analyzing, with a non-drug treatment, the protective and therapeutic effects of EVOO combined with high-fat and normocaloric diets, through an experimental model.

2. MATERIALS AND METHODS

2.1. Animals and experimental groups

This study complies with the ethical principles of animal experimentation, adopted by the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and Animal Experimentation of Evangelical College of Parana, record no. 10640/ 2014. This study was funded by Institutional Program of Scientific Initiation Scholarships (PIBIC), scholarship program of the National Council for Scientific and Technological Development (CNPq) - grant number 155148/2015-0.

Five-week-old male Wistar rats with an average weight of 230 g were obtained commercially from the Pontifical Catholic University of Parana Institution, in the city of Curitiba and were stored in the vivarium of the Evangelical College of Parana. During the 90-day experiment, all animals were housed in small polypropylene cages in a 12-hour controlled photoperiod (light/dark) at a temperature of 24 ºC ± 2 °C.

The animals were offered water *ad libitum*, and standard commercial diet for rats and mice (Presence®) containing 19% protein, 56% carbohydrate, 3.5% lipids, 4.5% fiber, 5.0% vitamins and minerals, totaling 17.03 kJ/g. The high fat diet, based on Duarte *et al*. 2006, was prepared by researchers and milled, mixed, sterilized, dried in an oven at 200 °C for 40 minutes and offered as pellets with the following proportions: 15 g standard feed, 10 g roasted peanuts, 10 g milk chocolate, 5 g cornstarch biscuits and 2.5 g egg yolks, containing 21% protein, 48% carbohydrate, 21% lipids, 4.0% fiber, 5.0% vitamins and minerals, totaling 55.15 kJ/g. A total of 20 g of feed per 200 g body weight for each animal was offered in both diets.

After one week of acclimation with water and *ad libitum* feed, rats were randomly divided into 6 groups of 3-5 rats (aiming at future replacement, refinement and reduction (3Rs) of the use of animals in research): Group NC (negative control: normocaloric diet), Group NEVOO (normocaloric diet and extra virgin olive oil), Group PC (positive control: high fat diet), Group HEVOO (high fat diet and extra virgin olive oil), group HF (high fat diet and extra virgin olive oil in fasting) and group DLO (dyslipidemia induction with high fat diet/treatment with extra virgin olive oil). According to the gavage technique, 0.5 ml of 0.9% physiological solution per 200 g of body weight was administered every day, late in the afternoon, to groups NC and PC. Extra virgin olive oil, containing 85.7% of lipids (84% of unsaturated fat), delta K ≤ 0.01 and ≤ 0.05% acidity, was also administered at the same amount to NEVOO and HEVOO groups. The HF group received EVOO after 10 hours of fasting. For the DLO group, 0.9% of physiological

solution was administered until day 45, and then extra virgin olive oil, similarly.

After 90 days of experiment, euthanasia was performed by direct puncture of left cardiac ventricle, on anesthetized animals. The anesthetic technique was intraperitoneal application of ketamine hydrochloride at a dose of 50 mg/kg of animal and xylazine hydrochloride at a dose of 10 mg/kg of animal.

2.2. Systemic parameters

During each week of the experiment, animals were weighed and had waist circumference measured. On day 01 and 45, blood samples were collected by venipuncture of the animal's tail. On day 45, venipuncture was performed in HEVOO group to obtain control data. On day 90, blood was collected by direct cardiac puncture of the left ventricle. This blood was used for determining the concentration of HDL, LDL, and total cholesterol and the final blood sample collected was used for performing haemogram analyses to check and exclude the possibility of parasitosis since parasitic diseases could influence the study.

At euthanasia, heart was removed for later weighing and measuring, and the carotid arteries, abdominal aorta and iliac vessels were removed for histopathological analysis. The organs and vessels were fixed in 10% formalin, and subsequently dehydrated in ethanol, soaked in xylene and finally embedded in paraffin wax. Paraffin blocks containing organs and vessels were sectioned (5 mm thick), and the cuts transferred to slides and stained with hematoxylin-eosin. Structure analysis was performed with an optical microscope (Nikon® E100).

2.3. Biochemical assessment

Blood was collected in heparinized tubes and centrifuged at 3500 rpm for 20 minutes and pipetted into the plasma. Blood count was performed and the analysis of HDL, LDL and total cholesterol concentration was based according to the instructions of LabTeste Diagnostica SA.

2.4. Assessment of vascular atherosclerotic lesions

The histological technique involves division of each vascular segment into 2-7 transverse pieces measuring 5 mm thick and staining them with hematoxylin and eosin (H&E). Assessment was made by a pathologist without prior knowledge of the slides according to the standard classification by vascular lesions Committee of the American Heart Association (Stary *et al.*, 1995) [16]:

Type I: thickening of the arteries' intima layer.

Type II: presence of foam cells.

Type III: small deposits of extracellular lipids

Type IV (atheroma): extracellular lipid core

Type V: fibrointimal thickening with or without calcifications

Type VI (advanced injury): plaque rupture and thrombosis.

2.5. Assessment of heart tissue

The heart was divided into transverse pieces measuring 3-5 mm thickness and was stained with H&E. The presence or absence of myocardial injury was rated according to Acikel, *et al*., 2005 [17]:

Type 0: No change.

Type 1: Small lesion with focal myocyte damage or small multifocal degeneration with mild inflammation.

Type 2: Moderate injury, with extensive myofibrillar degeneration and/or diffuse inflammatory process.

Type 3: Severe injury with necrosis and diffuse inflammatory process.

The heart tissue was also evaluated by the presence or absence of changes in coagulation necrosis areas, vacuolar degeneration, edema, loss of pyknosis cores and presence of lymphocytes permeating myocardial fibers and/or circulating necrotic cells according to Meyer, 2013 [18].

2.6. Statistical analysis

Results of quantitative variables were described as mean, median, minimum, maximum and standard deviation; qualitative variables were described as frequencies and percentages. For comparing quantitative variables between two groups, the non-parametric Mann Whitney test was used. The nonparametric Kruskal Wallis test was used when more than two groups were compared. To evaluate

the association between two dichotomous qualitative variables, the Fisher's exact test was used. Correlation between two quantitative variables was assessed by estimating the Spearman correlation coefficient. P values < 0.05 were considered statistically significant. Data were analyzed using Statistica software v.8.0.

3. RESULTS

3.1. Weight and abdominal circumference

The group that consumed normocaloric diet and olive oil presented lower mean weight compared to the group with hypercaloric diet and fasting olive oil (391 g *vs* 414.8 g, p = 0.0476). Regarding control groups, NEVOO showed lower weight means compared to NC and PC. HEVOO and DLO presented lower weight means than control groups. With regard to abdominal circumference, NEVOO showed lower abdominal circumference and weight, and HF presented the lowest mean abdominal circumference, but there was no significant difference (Figure 1).

3.2. Serum analysis

NEVOO presented lower values of total cholesterol compared to both control groups. Total serum cholesterol values were higher in the group consuming hypercaloric diet alone than in the group consuming hypercaloric diet with olive oil intake (83.36 mg/dL vs 77.51 mg/dL, p = 0.5179).

HDL-cholesterol levels were higher in the group that consumed normocaloric diet and olive oil compared to the group with hypercaloric diet and olive oil (52.61 mg/dL vs 42.83 mg/dL, p = 0.7857). In control groups, PC showed lower HDL-c values compared to NC and HF presented higher values when compared to PC and HEVOO.

NEVOO presented lower LDL values compared to control groups. Minor values were recorded in NEVOO group when compared to NC group (20.60 mg/dL vs 27.16 mg/dL, p = 0.3095). When olive oil was combined with hypercaloric diet, LDL values were higher when compared to PC group, demonstrating a possible ineffectiveness of this combination (26.83 mg/dL vs 23.80 mg/dL, p = 0.5714). The group with fasting olive oil intake presented a much lower mean in this analysis when compared to the other groups (16.20 mg/dL).

Regarding triglycerides (TRI), groups with normocaloric diet presented higher mean values due to the higher diet carbohydrate composition. There was significant difference between groups

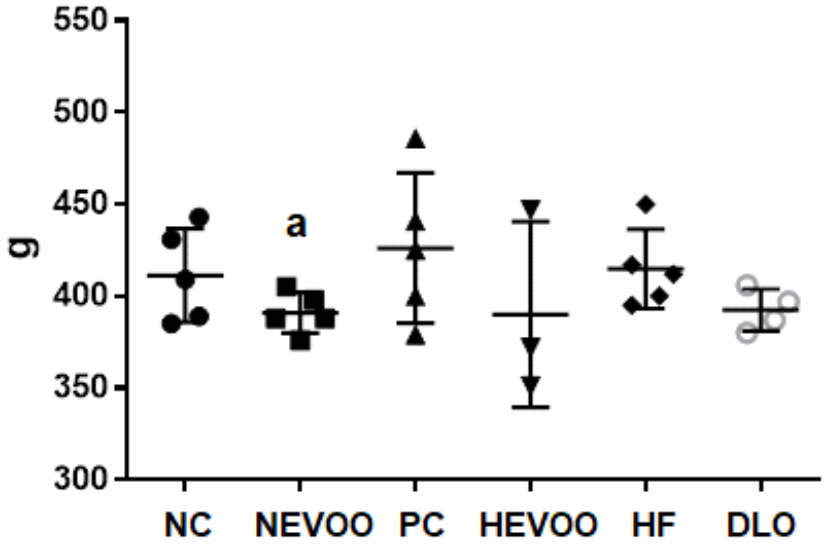

Figure 1. Body weight value of groups. Group NC (negative control: normocaloric diet); group NEVOO (normocaloric diet and extra virgin olive oil); group PC (positive control: high fat diet); group HEVOO (high fat diet and extra virgin olive oil); group HF (high fat diet and extra virgin olive oil in fasting); group DLO (dyslipidemia induction with high fat diet/treatment with extra virgin olive oil). [a]: P < 0.05 compared with HF group.

NC and HEVOO (31.04 mg/dL vs 152.9 mg/dL, p = 0.0357), and NC and DLO (31.04 mg/dL vs 91.66 mg/dL, p = 0.0159). DLO showed higher mean triglycerides values than PC (91.66 mg/dL vs 43.62 mg/dL, p = 0.0159, respectively).

The TRI/HDL index, a predictor of coronary disease (Gaziano *et al*., (1997) [19]), was significantly lower in the normocaloric diet group (0.6672) than in the group with dyslipidemia and subsequent ingestion of olive oil (2.265), p = 0.0159. The group that received olive oil along with normocaloric diet also had lower predisposition for coronary diseases compared to the group that received fasting olive oil (0.4704 *vs* 1.193, p = 0.0317) and to the DLO (0.4704 *vs* 2.265, p = 0.0159), demonstrating the importance of a balanced diet.

3.3. Cardiac tissue

3.3.1. Diameter and weight

There was no significant difference in heart diameter between groups. Regarding heart/body weight ratio, PC presented higher mean compared to NEVOO (p = 0.087). Among the other groups, the values remained constant.

3.3.2. Histology

Areas of vacuolar degeneration, edema, pyknosis, loss of nuclei and presence of lymphocytes were not found. Only one mouse from HF had areas of coagulation necrosis (subendocardial infarction in organization) and were classified as grade 3 of myocardial injury according to Aiken *et al.*, 2005 [20], histological scale (Figure 2).

3.4. Aorta, carotid and iliac arteries

In aorta histological analysis, all animals that consumed olive oil as treatment after induction of dyslipidemia showed grade II lesions, whereas only 20% of the animals in the group fed with normocaloric diet and olive oil throughout the experiment presented the same lesion degree (intimal thickening and presence of foam cells).

Regarding the histological analysis of the carotid artery, NC group presented 80% of the animals with grade II lesion, whereas NEVOO group showed only 40% of the animals with the same degree (both fed a balanced diet).

The analysis of the iliac artery found 20% of the animals of PC group with grade III lesion, and small extracellular lipid deposits, whereas in HF group, grade III lesion was not found in arteries (both with hyperlipid diet consumption).

3.5. Haemogram

Blood count was performed to rule out possible infections and parasites. There was no significant result.

4. DISCUSSION

Analysis of body weight showed significant difference between NEVOO and HF groups.

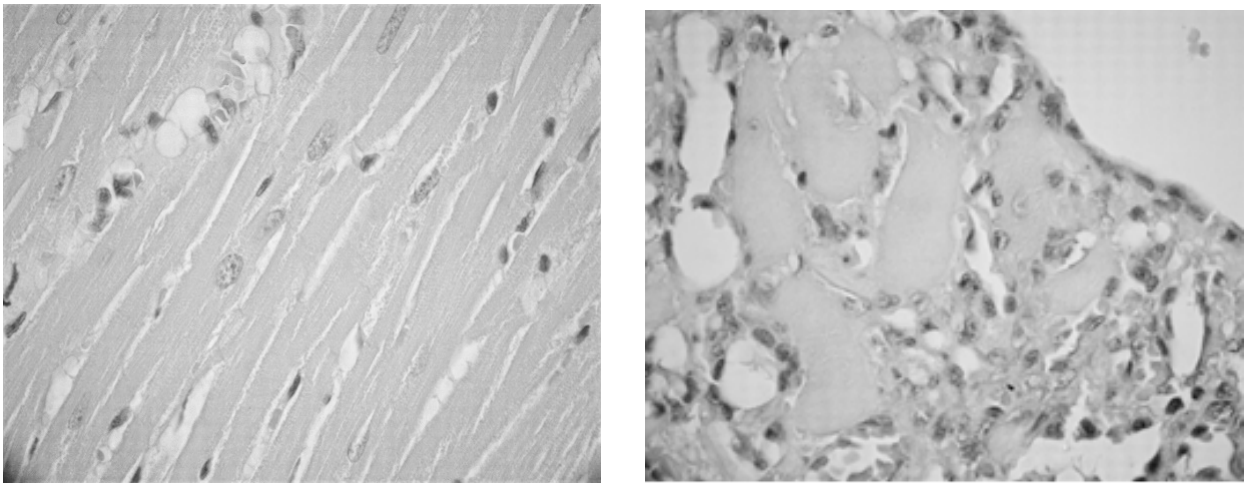

Figure 2. Normal myocardial histology (left) and myocardium with the subendocardial infarction hystology (right).

The fasting group showed weight gain likely because of consumption compensation of hypercaloric food before the period of food restriction. Regarding NC and NEVOO groups, the group that consumed extra virgin olive oil presented with lower mean body weight. Paniagua (2007) [21] and Karhunen (2008) [22] stated that oleic acid, the main representative of extra virgin olive oil, is an important monounsaturated fatty acid responsible for satiety control. Similarly to literature, DLO group presented lower body weight values compared to the group that did not consume extra virgin olive oil (positive control).

Prospective studies show that fat located in the abdomen is a risk factor for cardiovascular disease. Although the abdominal circumference did not present statistical difference with regard to control groups, the group that consumed the Mediterranean Diet presented lower abdominal circumference in the last measurement, similar to the findings in the literature [23].

According to Lopes, Peluzio and Hermsdorff (2015) [23], intervention studies indicate beneficial effects of habitual consumption of monounsaturated fatty acids from foods such as olive oil, markers of lipid metabolism, hypocaloric diets or consumption of other lipid sources. The effect can be positive with the improvement of the plasma lipid profile, either in concentration or in size of HDL-C and LDL-C particles, widely known as protection and risk factors, respectively, for cardiovascular diseases. Authors state that the mechanisms of action seem to be related to the effects on concentrations and particle size of lipoproteins and consequently on their metabolism at the cell level.

The lipid profile analysis confirms studies about the direct relationship between dietary pattern and the development of higher total and LDL-C levels. Oliveira *et al*. (2015) [24] state that the consumption of foods rich in refined carbohydrates, low in dietary fiber and with high levels of saturated fats increases the risk of developing cardiovascular diseases. On the other hand, the ingestion of vegetables and foods with high unsaturated fat (present in extra virgin olive oil) has a protective effect against the atherosclerotic process.

Currently, several studies suggest the use of an index, that was initially proposed by Gaziano *et al*., 1997 [19], determined from the patient's lipid profile and showing a strong correlation with cardiovascular risk: the triglyceride/HDL-cholesterol ratio (TRI/HDL), which strongly predicts the risk of acute myocardial infarction (AMI) and has been proposed as an easy-to-use atherogenic marker [25].

According to the findings of Maruyama *et al*., 2003 [26], the results indicate significant TRI/HDL value ratio corresponding to the presence of small and dense LDL particles (LDL particles for ratios > 1), referring to the groups that consumed hypercaloric diet compared to groups with a balanced diet. According to Li *et al*., 2015 [27], small and dense LDL particles are considered risk factors for cardiovascular disease. Thus, making use of estimates that can be calculated from the lipid profile, replacing high-cost specialized techniques such as non-denaturing gradient gel electrophoresis and nuclear magnetic resonance spectroscopy, is of great clinical and economic importance.

Analyzing the myocardial aspect, there was no significant macroscopic and microscopic differences. However, some researches such as Martins *et al*., 2015 [28], report the occurrence of myocardial hypertrophy and interstitial fibrosis, accompanied by functional disorders in experimental models such as those with diet-induced obesity. Oliveira-Junior *et al*., 2014 [29] state that saturated fatty acids are the main metabolic fuel for the heart and, antagonistically, the accumulation of excess lipids can stimulate mitochondrial overload and activate molecular mechanisms of cardiac remodeling. Da Silva *et al*., 2014 [30], on the other hand, observed that, although the increase in adipose tissue led to metabolic and hormonal changes, obesity did not result in cardiovascular morphology changes after 15 and 30 weeks, and remained stable during these two investigated periods.

According to arterial histological analysis, the groups that consumed olive oil presented a lower percentage of grade II arterial lesion in the carotid, aortic and iliac arteries. Kamalakkannan *et al*., 2016 [31] states that the high content of oleic acid in some oils, such as olive oil, causes

inhibition of atherosclerotic plaque formation, as well as suppression of testosterone synthesis, corroborating with that found in histological analyzes.

As quoted by Medina-Remón, 2017 [32], the cardiovascular protection of olive oil is attributed, in part, to the effects on factors for atherosclerosis. In this respect, a trend of atherosclerotic plaque reduction was observed in the carotid, iliac and aortic arteries, in the groups that had their diets supplemented with extra virgin olive oil, and in those animals, according to the scale used in the pathological evaluation, the maximum degree found was the degree 2, compared to grade 3 of the groups that consumed hypercaloric diets without oil supplementation. There is evidence that oils rich in polyphenols lower blood pressure and improve lipid profile, and have a significant effect of elevating HDL cholesterol. The beneficial effect of extra virgin olive oil on HDL-cholesterol is due, in part, to omega-9 and partially to polyphenols, which appear to have additional favorable effects on HDL functionality, promoting reverse cholesterol transport and also are implicated in the improvement of endothelial function [33].

The hydroxytyroses, compounds present in extra virgin olive oil, besides preventing the oxidation of low-density proteins, also play an important role in inhibiting endoplasmic reticulum stress in human cardiac and hepatic cells. This may be very relevant for the prevention of cardiovascular diseases because misplaced proteins and, therefore, dysfunctional lipoproteins are likely to be atherogenic through uptake by scavenger macrophage receptors. This action is very relevant for the prognosis of cardiovascular diseases, although it still needs to be confirmed in humans. The hydroxytyroses also stimulate lipolysis in pre-adipocytes contributing to the adjunct treatment of metabolic syndrome and related pathologies, namely obesity. These effects corroborate with the results of the present study where, notably, the improvement of risk factors for metabolic syndrome in animals supplemented with extra virgin olive oil is noticed [34].

5. CONCLUSION

The consumption of hypercaloric diet for 90 days caused significant changes in body weight. The association of extra virgin olive oil to the diet rich in saturated fats modified anthropometric data and lesions in the intima layer of the arteries in rats. Abdominal circumference, a highly predictive measure of cardiovascular diseases, although not statistically different, showed lower values for animals that consumed extra virgin olive oil.

The clinical management of hyperlipidemia is crucial to prevent coronary artery disease. The current results suggest that the continuous administration of a balanced diet plus extra virgin olive oil (Mediterranean Diet) has a potential positive effect against obesity, the lipid profile and consequently formation of atheroma plaque.

The detailed action of the compounds of extra virgin olive oil has been increasingly studied since the ingestion of unsaturated phenolic compounds has shown to be beneficial for reduction of the main risk factors for cardiovascular diseases, such as obesity and hypercholesterolemia, associated to a greater control of oxidative damage.

CONFLICT OF INTEREST STATEMENT

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

ABBREVIATIONS

AS	:	atherosclerosis
CVD	:	cardiovascular disease
DLO	:	dyslipidemia induction with high fat diet/treatment with extra virgin olive oil
EVOO	:	extra virgin olive oil
H&E	:	hematoxylin and eosin
HEVOO	:	high fat diet and extra virgin olive oil
HF	:	high fat diet and extra virgin olive oil in fasting
HDL-c	:	high-density lipoprotein cholesterol
LDL-C	:	low-density lipoprotein cholesterol
NC	:	negative control (normocaloric diet)
NEVOO	:	normocaloric diet and extra virgin olive oil
PC	:	positive control (high fat diet)
TRI	:	triglycerides

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