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DIETARY INTAKE AND BIOCHEMICAL RISK FACTORS FOR CARDIOVASCULAR DISEASE IN TWO RURAL REGIONS OF CRETE

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The aim of the present study was to identify any possible protective effects of wild greens on certain biochemical cardiovascular disease (CVD) risk factors in elderly women leaving in rural Crete. For the needs of the study a region with high consumption of wild greens (Avdou) and a region with low consumption (Anogia) were identified and a representative population from each area was recruited (n= 37 and n=35 respectively). Serum lipids and fibrinogen levels, total antioxidant capacity (TAC), soluble intercellular adhesion molucule-1 (sVCAM-1), soluble vascular adhesion molucule-1 (s-ICAM-1) and haematological factors were measured in both regions during winter and summer time when wild-green plants consumption is high and low, respectively. Regarding classic lipid risk factors for arteriosclerosis no significant different between the two regions were detected. TAC was found higher in Avdou compared to Anogia during winter screening, but null in both regions during summer. Fibrinogen was found higher in Avdou compared to Anogia while no differences between the two regions were detected for sVCAM-1 and s-ICAM-1 in both screenings. The significantly higher TAC and fibrinogen values detected in Avdou could be attributed to the higher wild green consumption in that region since resent data indicated that their content in vitamin C and E as well as flavonoids is particularly high.

Key words: *diet, serum lipids, fibrinogen, antioxidant capacity, adhesion molecules*

INTRODUCTION

The first epidemiological study revealing the beneficial effects of the traditional Cretan diet was the seven country study. In the middle of the previous century, Cretans were found to have the lowest rates of cardiovascular disease (CVD) and cancer, followed by the population of Japan (1, 2). The investigators concluded that the reason for those low rates must be the high olive oil intake and the low saturated fat intake of the Cretan diet. Such a dietary scheme providing high intake of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) and low intake of saturated fatty acids (SFA) could have favourable effects on lipid levels and consequently lowering the risk of CVD (1). However the protective effect of dietary fats could not provide sufficient explanation on the mortality rates from CVD, during the follow up of the seven country populations. Precisely, the mortality rate from CVD was lower in southern Europe compared to northern Europe but it was also much lower among the Cretan cohort compared to the other nine cohorts from the Southern Europe although the average cholesterol concentrations in the Cretan population were similar to those in the other Mediterranean cohorts (3).

What has been described in the literature as Mediterranean diet seems to be an amalgamate of certain components of the traditional Cretan diet, south Italian diet and Spanish Mediterranean regions diet. In most of the cases, Mediterranean diet has been described, either as dietary guidelines for the public or during controlled intervention studies, as a diet rich in olive oil, fruits, pulses, cereals and fish and replacement of meat with fish, poultry and vegetables (4). Based on scientific evidence such a diet or dietary guidelines can induce favourable changes in total cholesterol (TC), low density lipoprotein cholesterol (LDL) and blood pressure values among subjects with unfavourable levels but can not increase significantly the plasma antioxidants levels (4), or protect from LDL oxidation (5). The latest could be attributed to the lower level of antioxidant content of those food groups available in northern Europe compared to those available in south Europe or particular in Crete (3). Furthermore, this controversy could be attributed to miss-recorded or unintentionally neglecting the importance of certain food components of the traditional Cretan diet particular rich in polyphenols or antioxidant vitamins (5).

If we look back in the first available records describing traditional Cretan diet someone can realize that wild greens and herbs, food particular rich in polyphenol and antioxidants, had a central role in the traditional form of the diet. In 1948 traditional Cretan diet was described as „olives, cereals grains, pulses, wild greens, herbs and fruits, together with limited quantities of goat meat and milk and fish... no meal was completed without bread... olives and olive oil contributed heavily to the energy intake... food seemed to be „swimming in oil" (6). This is not exactly the profile of the Mediterranean diet as it has been until recently described in scientific journals or communicated to the public and this is probably because wild greens and herbs were incorrectly grouped under the wider group of vegetables. However, wild greens seem to be very different in polyphenolic and antioxidant content compared to cultivated vegetables (5, 7). The latest evidence on the further protective role of certain components and nutrients of the Mediterranean diet, further to olive oils and MUFAs, such as red wine, wild greens and herbs rich in polyphenols and antioxidants provide us with the unique opportunity to re-examine the theory of the protective role of the Mediterranean diet and focus our attention on the neglected role of these components and particular wild greens for which so little is known so far.

The aim of the current study was to record the possible protective role of these wild greens on certain CVD risk factors in a rural population following the Traditional Mediterranean diet as it was described by Keys (1) and Nestle (6); furthermore, to record and identify these plants and any traditional knowledge and health beliefs related to the consumption of these plants.

MATERIALS AND METHODS

Subjects and study design

For the purpose of the current study two regions of Crete, Avdou and Anogia were identified. These two regions were selected because they are both isolated and consequently not major changes on the lifestyle of the local populations have been noticed over the last few decades. The majority of the inhabitants of these two regions are low income elderly, all sharing the same culture, tradition and religion and their primary occupation is agriculture and farming. However, there is a difference between these two regions and this has to do primarily with the altitude and consequently the availability of the eligible wild green plans. The altitude in Avdou is relatively low and the flora is characterised by a big variety of wild greens while in Anogia, which is located at an altitude higher than 2,000 m, the variety in flora is limited. This fact has also an impact on the dietary habits of the populations leaving in these regions since the diet of the people in Avdou is richer in wild greens compared to Anogia.

Furthermore, since the primary aim of the current study was to compare the two populations regarding certain biochemical indices related to CVD, the confounding effect of other parameters, other than diet, had to be controlled. For this purpose the subjects recruited for the needs of the current study were elderly females. Elderly females in these rural areas of Crete are less luckily to be involved in heavy manual work, smoke or drink.

Subjects

Among the total population leaving in these two regions a random sample of 40 women was selected from each area. From those 80 women invited to participate in the study 37 from the area of Abvou and 35 from Anogia agreed to participate. All subjects were screened twice. The first examination took place during the time period of wild green plants consumption (February-March 2003) and the second measurement took place during summer time (August-September 2003), when wild greens were not available. The study was approved by the Bioethical Commitee of the Harokopio University.

Haematological and biochemical indices

Early morning venous blood samples were obtained from each subject for hematological and biochemical screening tests, following a 12-h overnight fast. Professional staff performed venipuncture, using vacutainers to obtain 20 ml of whole blood. Blood samples were transferred to the University Medical Hospital of Crete where hematological indices were measured and blood was centrifuged for plasma separation at 3,000 rpm for 15 min, using a bench centrifuge, and 1.5 ml aliquots were pipetted into plastic Eppendorf tubes. The aliquots were then stored in tanks containing dry ice that maintained the temperature at 3-4 °C and were transferred to the Department of Nutrition and Dietetics, Harokopio University, Athens for the completion of the biochemical indices. Plasma glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL) and triglycerides (TG) were determined in duplicate using commercially available enzymatic colorimetric assays (Sigma Diagnostics, St. Louis, MO) on an automated ACE analyser (Sciapparelli Biosystems, Inc., USA). LDL was calculated by the Friedewald equation [8].

For the fibrinogen levels evaluation, plasma was immediately loaded on a Coatron 2 analyzer (Biotechnology, Greece) and fibrinogen was measured using Teclot Fib. (W. Kaolin, TECO Medical In, G). For the quantitative determination of sICAM-1 and sVCAM-1, ELISA sandwich kits were used according to the manufacturer's instructions (R&D Systems, Inc., MN, USA). Similarly, for the estimation of the total antioxidant potential an ELISA assay kit, based upon the reduction of Cu^{++} to Cu^{+} by the combined action of all antioxidants present in the sample, was used according to the manufacturer's instructions (OxisResearch, PO, USA).

Anthropometric data

Body weight was measured by a digital scale (Seca) with an accuracy of ± 100 g. Subjects were weighed without shoes, in light clothing. Standing height was measured without shoes to the nearest 0.5 cm with the use of a commercial stadiometer, with the shoulders in relaxed position and arms hanging freely [9]. Body Mass Index (BMI) was calculated by dividing weight (kg) by height squared (m^2).

Dietary data and physical activity assessment

Dietary data was obtained with 24 hours recalls during morning interviews. Two interviews took place during weekdays and one over a weekend day. The dieticians, who were trained as a group to minimize inter-observer variation, completed a structured questionnaire for each subject after interviewing them individually. Particularly, subjects were asked to describe the type and amount of food as well as beverages consumed during the previous day. The interviewers used food models, designed by the National Dairy Council and specially prepared photographs of common and traditional Greek dishes of various portions, as well as household cups and measures to define amounts when appropriate. Food intake data was analyzed to macronutrients and micronutrients content using the Nutritionist V diet analysis software (First Data bank, San Bruno, CA). The data base was extensively amended to include traditional Greek recipes, as described in Food Composition Tables and composition of Greek cooked food and dishes (10, 11).

Statistical analysis

The analysis of data was conducted using the SPSS statistical software programme, Version 11. Continues variables are presented as mean values ± one standard deviation, while categorical variables are presented as absolute and relative frequencies. Continuous variables were tested for normality using the Kolmogorov-Smirnov criterion and skewed distributed variables were log transformed. Comparisons between normally distributed continuous variables were performed with the use of independent sample t-test while asymmetric continues variables were tested with the use of non parametric Mann-Whitney U test.

RESULTS

According to the descriptive data presented in *Table 1* there were no significant differences between women in Avdou and those in Anogia regarding their age, height, weight and BMI. Similarly, the majority of nutritional data obtained from these subjects were not found to differentiate significantly between the two regions (*Table 2*), neither at baseline examination in March nor at their follow-up re-examination in September.

Table 1. Anthropometric data from Avdou and Anogia region respectively.

Variables examined	**Avdou** (n = 37)	**Anogia** (n = 35)	***p*-value**
Age (yrs)	71.6 (±8.0)	68.9 (±6.2)	NS
Height (cm)	152.7 (±6.3)	152.7 (±4.5)	NS
Weight (Kg)	70.4 (±14.4)	71.7 (±12.8)	NS
BMI (Kg/m^2)	29.9 (±5.3)	30.5 (±5.0)	NS

BMI: body mass index

Table 2. Nutritional data as obtained with the use of the three days 24h-recall method from Avdou and Anogia region respectively.

		Avdou (n = 37)	**Anogia** (n = 35)	***p*-value**
Energy (kcal)	March	1801.7 (±649.3)	2131.0 (±1471.2)	NS
	September	1634.1 (±586.7)	1495.6 (±683.7)	NS
Protein (g)	March	42.6 (±25.9)	69.2 (±68.61)	0.039
	September	68.1 (±36.3)	74.7 (±38.1)	NS
Carbo (g)	March	183.3 (±66.1)	162.4 (±83.7)	NS
	September	174.2 (±58.2)	156.3 (±82.3)	NS
Total fat (g)	March	96.6 (±48.2)	116.5 (±94.2)	NS
	September	76.3 (±39.1)	74.0 (±28.1)	NS
SFA (% of energy)	March	10.3 %	15.8 %	0.045
	September	11.2 %	13.3 %	NS
MUFA (% of energy)	March	28.3 %	28.2 %	NS
	September	21.5 %	21.3 %	NS
PUFA (% of energy)	March	6.4 %	5.9 %	NS
	September	5.2 %	5.8 %	NS
Vit C (mg)	March	106.6 (±87.1)	216.2 (±904.9)	NS
	September	60.8 (±47.2)	56.9 (±23.8)	NS
Vit E (IU)	March	17.12 (±10.8)	18.6 (±15.8)	NS
	September	12.3 (±6.2)	11.6 (±4.4)	NS
Fiber (g)	March	18.9 (±12.0)	17.3 (±9.6)	NS
	September	13.9 (±8.4)	13.6 (±6.7)	NS

Carbo: carbohydrates; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids: PUFA: polyunsaturated fatty acids; Vit: vitamin

Hence the mean daily dietary intake of energy, carbohydrates and total fat, as well as of certain antioxidants (Vitamins C and E) and of fiber was found to be similar in women in Avdou and Anogia, respectively. The only exceptions were mean protein intake and the percent of energy derived from SFA in March, which were both found to be higher for women living in Anogia (69.2 g ± 68.6 vs. 42.6g ± 25.9, P=0.039 and 10.3% vs. 15.8%, P=0.045 respectively), compared to those from Avdou.

Table 3 summarizes the differences observed for certain haematological indices between women in Avdou and Anogia at both screening periods. Subjects from Avdou were found to have higher levels of RBC (4.4 ± 0,3 vs. 4.8 ± 0.4, P<0.001) and HCT (39.8 ± 2,8 vs. 42.2 ± 2.7, P=0.004), compared to women from Anogia, but only during the second reevaluation at September. No other significant differences were observed.

Table 4 presents the mean plasma values of certain biochemical indices, by region of residence (Avdou vs. Anogia) and period of evaluation (March vs. September). According to these data the plasma level of fibrinogen was found to be higher for women in Avdou both at March (432.4 ± 109.8 vs. 368.8 ± 82.7, P=0.010) and September (419.5 ± 100.9 vs. 336.8 ± 84.2, P=0.030), compared to women from Anogia. A similar finding was observed for TAC, which was also found to be higher for subjects screened at March in Avdou (0.165 ± 0.561 vs. 0.059 ± 0.287, P=0.001), compared to those in Anogia. TAC was null in both regions during September. No other significant differences were observed, regarding the data derived from the biochemical analysis.

Table 3. Hematological data from Avdou and Anogia region respectively.

		Avdou (n = 37)		**Anogia** (n = 35)		***p*-value**
RBC (K/μL)	March	4.5	(±0.4)	4.7	(±0.4)	NS
	September	4.4	(±0.3)	4.8	(±0.4)	0.0
HGB (mmol/L)	March	132.6	(10.5)	135.8	(12.3)	NS
	September	129.3	(11.2)	129.8	(10.4)	NS
HCT (%)	March	40.7	(±3.2)	40.5	(±3.4)	NS
	September	39.8	(±2.8)	42.2	(±2.7)	0.004
LYM (K/μL)	March	2.1	(±0.6)	2.3	(±1.4)	NS
	September	2.5	(±0.6)	2.2	(±1.5)	NS
GRAN (K/μL)	March	3.5	(±0.9)	3.5	(±0.9)	NS
	September	3.6	(±1.0)	3.3	(±0.9)	NS
PLT (K/μL)	March	223.0	(±67.3)	236.1	(±48.7)	NS
	September	216.2	(±62.3)	240.6	(±50.5)	NS

RBC: red blood cells; HGG: haemoglobin; HCT: haematocrite; LYM: lymphocytes; GRAN: granulocytes; PLT: platelets.

Table 4. Biochemical data from Avdou and Anogia region respectively

		Avdou (n = 37)	Anogia (n = 35)	*p*-value
TC (mg/dL)	March	247.7 (±41.9)	248.2 (±40.3)	NS
	September	249.0 (±42.0)	250.0 (±41.1)	NS
TG (mg/dL)	March	144.2 (±53.1)	140.5 (±46.6)	NS
	September	150.7 (±62.9)	139.4 (±47.9)	NS
HDL (mg/dL)	March	47.6 (±10.4)	50.8 (±10.3)	NS
	September	48.4 (±9.7)	50.7 (±9.7)	NS
LDL (mg/dL)	March	172.7 (±41.4)	168.2 (±34.9)	NS
	September	170.9 (±34.4)	174.5 (±34.9)	NS
Fibrinogen (mg/dL)	March	432.4 (±109.8)	368.8 (±82.7)	0.010
	September	419.5 (±100.9)	336.8 (±84.2)	0.030
TAC (mM Uric Acid)	March	0.165 (±0.561)	0.059 (±0.287)	0.001
	September	0	0	NS
VCAM-1 (ng/dl)	March	738.8 (±166.7)	767.1 (±231.2)	NS
	September	766.3 (±218.9)	863.1 (±260.1)	NS
ICAM-1 (ng/dl)	March	281.7 (±47.5)	261.5 (±57.9)	NS
	September	273.9 (±49.3)	251.2 (±40.3)	NS

TC: total cholesterol; TG: triglycerides; HDL: high density lipoprotein; LDL: low density lipoprotein; TAC: total antioxidant capacity; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intercellular adhesion molecule 1

DISCUSSION

The findings of the present study revealed that the percentage contribution of fat in total energy intake in both regions examined showed a seasonality variation, which has not been reported or examined previously. The mean contribution of fat to the total energy intake was found to be 48.6% and 49.2% in Avdou and Anogia respectively during wintertime. A decline was observed in summer with average values becoming 42.0% and 44.5% in Avdou and Anogia respectively. These figures are higher than that reported for the traditional Cretan diet in the seven countries study (40%) and higher to the mean values reported by Moschandreas and Kafatos (12) for a similar age urban population in Crete (39.8%). This should be attributed to the type and amount of food consumed around the year and the climatic conditions affecting the food choices of the two populations. During winter time consumption of meat and dairy products increases particularly in Anogia while consumption of wild greens, easily accessible, cooked with a lot of olive oil are consumed in Avdou. These differences provide some explanation on the higher intake of protein and SFA detected in Anogia, compared to Avdou, during wintertime.

Changes in the diet of contemporary Cretans compared to those in 1960 has also been observed for the contribution of SFA, MUFA and PUFA in the diet. In the traditional Cretan diet SFA was contributing less than 10% of the total energy intake, while the intake of MUFA was very high (about 80g/day) (5). In the current study the percentage contribution of SFA in the Avdou region is closer to the traditional diet (10,3% and 11,2% during winter and summer time respectively). In Anogia the figures are quite different for SFA (15.8% and 13.3% in winter and summer time respectively) and much closer to that reported by Moschandreas and Kafatos (12) for contemporary Cretans living in urban areas and being at the highest quartile of fat intake.

Regarding blood indices, all hematological markers were within normal limits, while significantly higher RBC and HCT values were observed during summer time screening in the Anogia region. Since these indices are sensitive to iron intake the observed differences could be attributed to the higher meat consumption in Anogia compared to Avdou during the previous months. One more parameter possibly contributing to the observed hematological differences is the higher altitude in the Anogia region (13). Regarding biochemical indices, the mean values of TC and LDL as well as the atherogenic index of TC/HDL are above recommended levels. Although SFA intake was found to be lower in Avdou during wintertime screening this was not followed by lower lipid values. The lack of differences in serum lipids could be probably explained by the lack of differences in MUFA and PUFA intake in both screenings while no differences were observed for SFA during summer time.

TAC values were found to be significantly higher in Avdou compared to Anogia during winder screening, a finding that is not in agreement with the dietary data where no differences were detected in vitamin E and C intakes. Still, the dietary data findings cannot exclude the possibility that such differences might exist but were undetected in the current study. The same applies for the flavonoid content of the diet in the two regions, which was not estimated due to the limited data on the consumption of many local foods, particularly wild greens. Probably the differences detected in TAC could reflect differences in antioxidants, vitamins and flavonoid intakes between the two regions, which were not detectable by the dietary analysis data. This is a limitation of the current study and it is primarily connected to our limited knowledge on the composition of these locally consumed wild greens. This is in line with the observation of Kok and Kromhout (5), indicating that is more likely that all these years the intake of flavonoids and total antioxidants in the Cretan population has been underestimated. High intake of dietary antioxidants potentially elevates total plasma antioxidants and this has been inversely correlated with risk for CVD (14, 15).

Recent data indicate that wild greens are naturally rich in vitamin E and C (16) and flavonoids and polyphenols, since these are metabolites of plant secondary metabolite. Because polyphenols have been found with antioxidative properties (17), these minor compounds also contribute to the antioxidant capacity of wild greens. In many cases the flavonoid content of these plants is much higher compared to those detected in cultivated vegetables, like onions which are known for their high flavonoid content (7).

Fibrinogen is affected by race and its levels increases with age, smoking, use of oral contraceptives and menopause and decrease with alcohol intake, exercise and hormone replacement therapy (18). In principle, all these variables were equal between the two populations and the observed differences could not be attributed to any of those. Regarding diet, the available data in the literature on the possible role of PUFA, MUFA or SFA on fibrinogen levels are still controversial. The available studies indicating a decrease in fibrinogen with increased consumption of long chain n-3 fatty acids are as many as those showing no effect while there are at least two showing an increase (19, 20). Furthermore, the replacement of SFA with MUFA and PUFA seems to increase fibrinogen (20) while the ratio of n-6 to n-3 seems to be the crucial factor in determining fibrinogen levels. The recommended ratio of n-6 to n-3 fatty acids in the diet has been set between 5:1 and 10:1 but probably the Cretan diet is much more rich in n-3 fatty acids. Meat, cheese, eggs and snails in Crete have a high concentration of n-3. For instance, the ratio of n-6 to n-3 in Cretan eggs has been found to be 1.3 where the USDA egg has a ratio of 19.4 (21). All these products are coming from animals that grazed rather than being fed grain and consequently a major constituent of their diet is wild greens. Wild greens which are also a major component in the diet of the local populations, seem to be very high in n-3 fatty acids (22) and contain higher amounts of alpha-linolenic acid than cultivated plants (16). The high content of n-3 of these plants could possibly explain the higher values of fibrinogen observed in Avdou compared to Anogia. However since limited data exists on the composition of the large variety of wild greens consumed by the locals only assumptions can be made until more data on the profile of these plants will be available in order safe conclusions to be reached.

Based on the available literature sVICAM and sICAM could be significantly lowered by diet alterations leading to increase intake of antioxidants and polyphenols in the diet of hyperlipidemic patients (23-25). In the current study no differences detected on sVICAM and sICAM levels of the two populations although probably the diet of the Avdou region was higher in antioxidants and polyphenols due to the higher intake of wild greens. This could be probably attributed to the fact that the diet of the two populations is already rich in antioxidants and polyphenols (due to olive oil, fruit and vegetables, other than wild greens) and the speculated higher intakes in Avdou could not induce any further changes. This is also supported by the relatively low sVICAM and sICAM values observed in both populations despite their high BMI values and unfavorable lipid profile. (23, 24).

The high serum lipid levels observed in the current study are in line with previous reports indicating that although Cretans have low mortality rates from CVD their serum lipids levels are comparable to northern Europeans (3, 26). Further more the increased BMI values observed in the current study seems to be the outcome of a more sedentary lifestyle adopted by the local populations over the last decades with improved living conditions, transportation and induced changes in agricultural and farming procedures. Both regions examined in the current study have a high intake of fat, primarily due to increased consumption of olive oil, while the higher values detected in TAC and fibrinogen in Avdou could be attributed to higher wild green consumption. Still our limited knowledge on the composition of these plants as well as the small number of subjects recruited in the current study can not lead to safe conclusions but certainly indicates the need for further research in this field and expansion of our knowledge on the use of local foods and wild greens as well as on their chemical composition.

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