

## SERUM LIPIDS, APOPROTEINS AND NUTRIENT INTAKE IN RURAL CRETAN BOYS CONSUMING HIGH-OLIVE-OIL DIETS

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**Abstract**—A high intake of olive oil has produced high levels of high-density and low levels of low-density lipoprotein cholesterol in short-term dietary trials. To investigate long-term effects of olive oil we have studied the diet and serum lipids of boys in Crete, where a high olive oil consumption is the norm.

Seventy-six healthy rural Cretan boys aged 7-9 years were studied. The diet was assessed by a 2-day dietary recall. Blood was collected according to a standardized protocol and sera were analyzed in a rigidly standardized laboratory. The mean daily intake of energy was 11.0 MJ (2629 kcal). The intake of fat (45.0% of energy) and oleic acid (27.2% of energy) was high, and that of saturated fat low (10.0% of energy), reflecting a high consumption of olive oil. The high consumption of olive oil was confirmed by a high proportion of oleic-acid (27.1%) in serum cholesteryl fatty acids. Mean concentration of serum total cholesterol was 4.42 mmol l<sup>-1</sup> (171 mg dl<sup>-1</sup>), of HDL-cholesterol 1.40 mmol l<sup>-1</sup> (54 mg dl<sup>-1</sup>), of serum triglycerides 0.59 mmol l<sup>-1</sup> (52 mg dl<sup>-1</sup>), of apo-A, 1210 mg l<sup>-1</sup> and of LDL apo-B 798 mg l<sup>-1</sup>. The body mass index of the Cretan boys (18.2 kg m<sup>-2</sup>) was on average 2 kg m<sup>-2</sup> higher than that of boys from other countries. Contrary to our expectation, the Cretan boys did not show a more favourable serum lipoprotein pattern than boys from more westernized countries studied previously using the same protocol. Our hypothesis that a typical, olive-oil-rich Cretan diet causes a relatively high HDL- to total cholesterol ratio is not supported by the present findings.

Diet Olive oil Serum lipids Apolipoproteins Fatty acids Boys

### INTRODUCTION

Reduction of serum cholesterol levels through diet [1] or drugs [2] will lower the risk for coronary heart disease (CHD). However, there is uncertainty as to which dietary changes should be recommended, because both traditional fat-modified diets and the recently favoured low-fat, high-carbohydrate diet tend to lower the protective high-density lipoproteins (HDL) along with the atherogenic low-density lipoproteins (LDL)

[3-5]. For this reason there is great interest in recent reports which indicate that in short-term controlled experiments, diets high in mono-unsaturated fatty acids will lower LDL but not HDL [4, 5]. One would like to support such observations with epidemiological data, because these reflect the long-term effects of diets consumed in actual practice. A problem in such studies is that in cross-cultural comparisons between adult populations the effect of diet on the cholesterol concentration in the high-density lipoproteins can be obscured by effects of other factors known to affect HDL, such as physical activity [6], body fatness [7], and use of tobacco [8], alcohol [9] and drugs [10]. This may explain why overall differences in HDL in adult men

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from different populations are nil [11, 12] or small [13]. However, clear-cut positive relations between HDL and diet have been observed in international studies of young boys [14–16]. At that age, obesity, lack of physical activity and used tobacco, alcohol and drugs are usually absent and cannot obscure the effect of dietary differences. We have previously studied boys from populations with high or low total fat intake, and have observed relations between total fat intake and serum HDL- and total cholesterol that agreed very well with data from short-term controlled trials. We have now studied boys from Crete, who traditionally have a high intake of monounsaturated fatty acids in the form of olive oil. The hypothesis to be tested was that the Cretan boys have a relatively high HDL-cholesterol to total cholesterol ratio, due to a diet high in total fat but low in saturated fatty acids.

#### METHODS

The study was carried out in the spring of 1986 in small rural villages in the central part of Crete, southeast of Iraklion. In the study area all healthy boys between 7 and 9 years of age ( $n = 97$ ) participated in the study. They came from 18 schools in the following villages: Agia Paraskevi ( $n = 2$ ), Amariano (5), Apostoli (5), Archangelos (4), Armaha (2), Aski (4), Diavaide (2), Evangelismos (7), Geraki (8), Jofori (4), Kasteli (14), Kastamonitsa (10), Liliano (2), Mathia (1), Polithea (8), Samba (3), Voni (5) and Xidas (11). Before the boys were asked to participate, their parents and the local teachers were contacted and informed about the aim and design of the study. The parents gave their written permission for their sons to participate. Height and weight of each boy were measured and information was obtained on alcohol, tobacco and drug use, on recent illness, on the father's occupation and on any infractions on the prescribed 14-hr fast before blood letting. A fasting blood sample was drawn according to the same rigidly standardized protocol used in previous international studies from Wageningen [15, 16]. After separation of the serum from the blood cells, sera were stored at  $-20^{\circ}\text{C}$  until shipment in the frozen state by air express to the Netherlands. All samples arrived frozen and were stored at  $-80^{\circ}\text{C}$  until analyzed. Materials needed for the drawing of blood and for the storage and transport of serum were brought from the laboratory in Wageningen during a field visit

by MBK prior to the study. The protocol for the selection of subjects, sampling and handling of blood, and production and dispatching of serum were thoroughly discussed at that time, and the need for the boys to be fasting was stressed during preliminary visits to the schools. As the objective of the study was to examine the effect of long-term consumption of olive-oil on serum lipoproteins rather than to collect representative data for Cretan children, strict random samples were not required.

Food consumption of each boy was estimated on two consecutive days. One of us (BC) met with the mothers and explained how to record the food intake of the children and provided diet scales. Every mother was also visited at home to observe her food preparation methods, and to measure cups, glasses and spoons. The records were collected at the homes and reviewed with the mothers to clarify any ambiguities present. Boys were asked individually what they had consumed outdoors. Duplicates of these items—usually prepacked commercial products—were subsequently bought and weighed by us. Foods were coded and nutrients calculated at the University of Crete, using the USDA food composition table adapted for local use. For foods which were consumed regularly by the boys and for which no nutrient data were available, duplicates were collected on the spot and analyzed in Wageningen, and the results incorporated into the food composition table. This involved home-made cheeses and typical Greek dishes like fasolada, dolmades, mousaka and souvlaki.

As required by our international protocol [15, 16] the concentration of C-reactive protein was measured as an indicator of infectious disease at the University of London by Professor M. B. Pepys, and that of albumin as an indicator of protein nutritional status. The presence of chylomicrons in serum was checked both by electrophoresis on cellulose acetate strips (Cellogel OIA52-25, Chematron, Milan, Italy) and by microcentrifugation [17]. A boy was considered as fasting only when no chylomicrons could be detected by either method and no consumption of food was reported over the last 14 hr. Data from 21 boys were eliminated before the analysis of the results because of levels of C-reactive protein exceeding  $10\text{ mg l}^{-1}$  ( $n = 7$ ), non-fasting state ( $n = 11$ ), recent illness ( $n = 1$ ) or missing data ( $n = 2$ ). None of the boys had levels of albumin less than  $36\text{ g l}^{-1}$ , except for the boy who also reported recent illness. Sera were analyzed for total and HDL-cholesterol and

Table 1. Physical characteristics and serum C-reactive protein and albumin levels of 76 healthy 8–10 year old boys from rural Crete in 1986

Characteristic	Mean	SD	Range
Age (months)	108.8	8.5	95.0–123.0
Height (cm)	133.2	6.2	118.5–148.2
Weight (kg)	32.5	5.6	24.6–49.5
Body mass index (kg m <sup>-2</sup> )	18.2	2.4	13.4–26.1
C-reactive protein (mg l <sup>-1</sup> )	2.2	1.9	1.0–10.0
Albumin (g l <sup>-1</sup> )	43.4	2.5	36.7–48.6

triglycerides, using enzymatic methods and strict quality control [18–21]. The within-run CV for control sera was 0.9% for total and 1.8% for HDL-cholesterol and 1.0% for total triglycerides. Accuracy was checked by analysis of serum pools of known value provided by the Centers for Diseases Control (Atlanta, Ga). Mean bias with regard to CDC-target values was +0.1% for total cholesterol, –3.2% for HDL-cholesterol and –1.5% for total triglycerides. The LDL-cholesterol concentration was calculated using the Friedewald equation [22]. The concentrations of total serum apolipoprotein-A<sub>1</sub> and LDL apoprotein-B were measured by radial immunodiffusion. Goat antiserum against apolipoprotein-A<sub>1</sub> and rabbit antiserum against apolipoprotein-B were kindly provided by Dr L. M. Havekes from the Gaubius Institute, Leiden, The Netherlands. Calibration standards were prepared from serum pools of known value provided by the Centers for Diseases Control. The combined within-day and between-day coefficients of variation for control sera was 4.6% for apolipoprotein-A<sub>1</sub> and 5% for LDL apolipoprotein-B.

Aliquots of serum were pooled for analysis of the fatty acid composition of cholesteryl esters. Each of the 17 pools consisted of equal amounts of serum of four boys. For eight boys not enough serum was available for this analysis. Physical characteristics, serum parameters and nutrient intake of these eight boys were not different from the other 68 boys, except for the HDL- to LDL-cholesterol ratio ( $0.45 \pm 0.10$  vs  $0.55 \pm 0.21$ ,  $p < 0.05$ ). The fatty acid composition of the cholesteryl esters was determined after extraction of lipids from a 0.6 ml aliquot of serum as earlier described [23, 24]. The proportions of the individual fatty acids were expressed as proportion by weight of all detected fatty acids. Also, oleic acid was expressed as proportion by weight of the six principal fatty acids (C16:0, C16:1, C18:0, C18:1, C18:2, and C20:4), which accounted on average for 93.7%

of the total cholesteryl ester fatty acids, to make a comparison possible with the data of Knuiman *et al.* [24].

Statistical analyses were carried out at Wageningen Agricultural University with the Statistical Package for the Social Sciences [25]. Dietary variables were calculated as individual means over 2 days and then averaged over the boys. Serum triglyceride and LDL apolipoprotein-B values were transformed into their natural logarithm in order to reduce skewness and kurtosis. Pearson correlation coefficients were computed between selected variables. Analysis of covariance was used to estimate the effect of selected dietary variables on serum parameters, with body mass index as a covariable. For all analyses two-tailed tests of statistical significance were applied, and a  $p$ -value of less than 0.05 was considered as statistically significant.

## RESULTS

Table 1 shows some characteristics of the study population. Most of the 76 boys were 8 or 9 years old. Their fathers were mainly farmers and manual workers. The Cretan boys were nearly as tall (133 cm) as their counterparts from other Western-European countries and the U.S.A. (133–138 cm), but the mean weight of the Cretan boys was about 2.5 kg higher [15, 26]. None of the boys reported alcohol use or smoking. Table 2 shows that the intake of saturated fatty acids was low and that of oleic acid quite high, due to a liberal consumption of olive-oil. Typical dishes eaten were potatoes and eggs fried in olive oil, and bread dipped in liberal amounts of oil. Table 3 shows the fatty acid composition of the serum cholesteryl esters. The high intake of olive oil is reflected in the linoleic-to oleic-acid ratio of the cholesteryl esters, which

Table 2. Nutrient intakes of 76 healthy 8–10 year old boys from rural Crete in 1986, as determined by 2-day recalls

	Mean	SD	Range
Energy (MJ)*	11.0	1.9	6.0–17.2
Protein (% of energy)	10.9	1.8	7.5–17.2
Carbohydrate (% of energy)	44.3	6.5	32.7–61.4
Total fat (% of energy)	45.5	6.2	28.0–56.6
Saturated fatty acids (% of energy)	10.0	2.4	5.2–16.2
Oleic acid (% of energy)	27.2	4.7	12.8–36.0
Linoleic acid (% of energy)	3.4	1.2	1.2–7.2
Cholesterol (mg day <sup>-1</sup> )	353	192	20–825
Fiber (g day <sup>-1</sup> )	18.4	7.8	6.2–42.7

\*1 MJ = 239 kcal.

Table 3. Composition of serum cholesteryl fatty acids (g 100 g<sup>-1</sup> fatty acid methyl ester) in pooled serum samples (n = 17) of 68 healthy 8–10 year old boys from rural Crete in 1986

Fatty acid	Mean	SD	Range
C14:0	2.1	1.1	0.9–4.8
C16:0	10.8	0.6	9.5–11.7
C16:1 (n – 7)	2.2	0.5	1.4–3.7
C18:0	0.6	0.2	0.0–0.8
C18:1 (n – 9)	27.1	1.8	23.9–30.7
C18:2 (n – 6)	47.3	2.7	40.7–51.5
C18:3 (n – 3)	0.7	0.3	0.3–1.3
C20:4 (n – 6)	5.7	0.9	4.2–7.1
Others	3.6	3.0	1.3–10.8

is much lower than that of boys from other countries [24]. Serum lipids and lipoproteins are given in Table 4 and Fig. 1. Mean total and HDL-cholesterol values (Fig. 1) were quite similar to those of other European boys studied in our recent international study [16] and of American boys studied by equivalent methods in the Lipids Research Clinics Prevalence Study in the early 70s [26].

The percentage of energy from oleic acid was positively correlated with the body mass index ( $r = 0.24$ ,  $p < 0.05$ ). Dietary fiber intake was negatively related to the LDL-cholesterol to apo-B ratio ( $r = -0.32$ ,  $p < 0.01$ ), even after adjusting for body mass index. No other significant relationships were found of any of the dietary variables with serum parameters by either correlation analysis or analysis of covariance.

Table 5 shows the correlations among lipid and lipoprotein variables. Strong positive correlations were observed between apo-A<sub>1</sub> and HDL-cholesterol, between LDL apo-B and total and LDL-cholesterol, and between the HDL-cholesterol to LDL-cholesterol ratio and the apo-A<sub>1</sub> to LDL apo-B ratio. This suggests that there was rather little variation in the composition of HDL and LDL in our sample,

Table 4. Serum lipids, lipoproteins and apoproteins in 76 healthy, fasting 8–10 year old boys from rural Crete in 1986

	Mean	SD	Range
Total cholesterol (mmol l <sup>-1</sup> )*	4.42	0.65	3.16–6.77
HDL-cholesterol (mmol l <sup>-1</sup> )*	1.40	0.28	0.84–2.31
LDL-cholesterol (mmol l <sup>-1</sup> )*	2.75	0.62	1.40–5.32
HDL-/total cholesterol ratio	0.32	0.07	0.17–0.61
HDL-/LDL-cholesterol ratio	0.54	0.20	0.22–1.65
Triglycerides (mmol l <sup>-1</sup> )†	0.59	0.34	0.16–2.29
Apolipoprotein-A <sub>1</sub> (mg l <sup>-1</sup> )	1210	206	891–1740
LDL apolipoprotein-B (mg l <sup>-1</sup> )	798	175	324–1395

\*1 mmol l<sup>-1</sup> = 38.7 mg dl<sup>-1</sup>.

†1 mmol l<sup>-1</sup> = 88.5 mg dl<sup>-1</sup>.

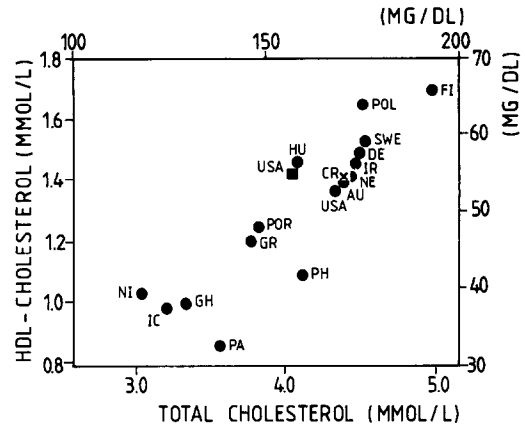


Fig. 1. Serum total and HDL-cholesterol in healthy 7–10 year old boys from Crete (x) compared with boys from different populations studied by us previously (●) and with 5–9 year old white boys from the U.S. Lipid Research Clinic Prevalence Study Visit II (■) [26]. Modified with permission from data of Knuiman *et al.* [35]. AU, Austria; CR, Crete; DE, Denmark; FI, Finland; GH, Ghana; GR, Greece; HU, Hungary; IC, Ivory Coast; IR, Ireland; NE, The Netherlands; NI, Nigeria; PA, Pakistan; PH, The Philippines; POL, Poland; POR, Portugal; SWE, Sweden; U.S.A., United States of America.

and that the measurement of apolipoproteins added little extra information. Only the HDL-cholesterol to apo-A<sub>1</sub> ratio correlated with body mass index ( $r = 0.28$ ,  $p < 0.05$ ).

The 21 boys whose data were excluded from the analysis, had, of course, a higher mean C-reactive protein level ( $10.3 \pm 14.1$  mg l<sup>-1</sup>) than the other boys, because this was an exclusion criterium. Most of their other characteristics were similar to those of the healthy, fasting boys (e.g. body mass index:  $17.4 \pm 2.5$  kg m<sup>-2</sup>; total cholesterol:  $4.26 \pm 0.60$  mmol l<sup>-1</sup> or  $165 \pm 23$  mg dl<sup>-1</sup>; HDL-cholesterol:  $1.30 \pm 0.23$  mmol l<sup>-1</sup> or  $50 \pm 9$  mg dl<sup>-1</sup>). Mean serum triglycerides were higher ( $0.68 \pm 0.37$  mmol l<sup>-1</sup> as opposed to

Table 5. Univariate Pearson correlation coefficients between serum apo-A<sub>1</sub>, LDL apo-B and their ratio and serum lipids and the HDL- to LDL-cholesterol (HDL-C/LDL-C) ratio in 76 healthy 8–10 year old boys from rural Crete in 1986

	Apo-AI	LDL-apo-B	Apo-AI/LDL-apo-B
Total cholesterol	0.30**	0.81***	-0.35**
LDL-cholesterol	-0.02	0.91***	-0.59***
HDL-cholesterol	0.81***	-0.23*	0.64***
Triglycerides	-0.18	0.23*	-0.32**
HDL-/LDL-cholesterol	0.52***	-0.70***	0.91***

\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

$0.59 \pm 0.34 \text{ mmol l}^{-1}$  ( $60 \pm 14$  and  $52 \pm 13 \text{ mg dl}^{-1}$ , respectively) for the fasting boys), which confirms the validity of the tests applied to identify non-fasting subjects.

## DISCUSSION

### *Cholesterol*

The main hypothesis to be tested in this study was that boys in rural Crete would have higher HDL- to total or HDL- to LDL-cholesterol ratios than boys in other populations because of a high consumption of olive oil. Therefore, a comparison was made of mean serum lipid values of 76 healthy Cretan boys with those of boys from other populations. These latter data were obtained by Knuiman *et al.* [14, 15] and Sullivan *et al.* [16] at our laboratory, using exactly the same protocol and methodology. The diet of the Cretan boys was high in total and monounsaturated fat and low in saturated fat, due to a liberal use of olive oil. In the respect they are similar to their grandfathers' [27] and fathers' (Aravanis *et al.*, unpublished results) generation. The composition of serum cholesteryl esters reflects the dietary fat composition of preceding weeks [28]. The proportion of oleic acid in the cholesteryl esters of the Cretan boys (28.9%) was appreciably higher than that in 7 and 8 year old boys from Western countries (20–26%) [24]. This validates the dietary data and shows that consumption of olive oil is high in these children. As expected, their HDL-cholesterol was comparable to that in boys from other European populations or the U.S.A. [14–16, 26]. Contrary to expectation, their total and LDL-cholesterol were not appreciably lower. In fact, serum total cholesterol levels in Crete were significantly higher than in the U.S.A. [26] as indicated by the 95% confidence interval for the difference in mean cholesterol levels ( $0.24$ – $0.56 \text{ mmol l}^{-1}$ , or  $9.2$ – $21.6 \text{ mg dl}^{-1}$ ). They were very similar to those in the Netherlands [15] (95% confidence interval:  $-0.28$ – $0.12 \text{ mmol l}^{-1}$ , or  $-10.8$ – $4.6 \text{ mg dl}^{-1}$ ).

There is no satisfactory explanation for this unexpected finding. One might speculate that the effect of monounsaturated fatty acids on serum lipids, found in controlled trials [4, 5] is transient. Epidemiological studies [15, 16] suggest that at least part of the effect of total fat intake on HDL-cholesterol and triglyceride levels is permanent. However, to our knowledge, no specific epidemiological information on the effect of monounsaturated fatty acids on LDL-

cholesterol is available as yet. For an alternative explanation one could turn to the body mass index. The mean body mass index of the Cretan boys ( $18.2 \text{ kg m}^{-2}$ ) was higher than that of comparable boys in other European countries ( $15.8$ – $16.8 \text{ kg m}^{-2}$ ) [15] or of American boys ( $16.5 \text{ kg m}^{-2}$ ) [26]. In young male adults the body mass index correlates positively with LDL-cholesterol [29, 30]. However, no association between body mass index and LDL-cholesterol is generally observed in children [30]. Thus, body mass index does not provide a satisfactory explanation either, and the cause of the apparent discrepancy between diet and LDL-cholesterol in the Cretan boys remains unclear.

### *Apolipoproteins*

Alaupovic [31] reported apo-A<sub>1</sub> levels of  $130 \text{ mg dl}^{-1}$  and total apo-B levels of  $72 \text{ mg dl}^{-1}$  in American children aged between 5 and 9 years. If one takes into consideration that most of the apo-B in plasma is bound to LDL then these values are close to our values ( $1210$  and  $798 \text{ mg l}^{-1}$ , respectively). In contrast, Solakivi-Jaakkola [32] reported a mean apo-A<sub>1</sub> concentration of  $160 \text{ mg dl}^{-1}$  and mean apo-B concentrations of  $93 \text{ mg dl}^{-1}$  in 9-year-old Finnish boys. These values are considerably higher than those found here. From other studies it is known that Finnish children have higher total and HDL-cholesterol concentrations than children in other countries [14]. In view of the high correlation between apo-A<sub>1</sub> and HDL-cholesterol, and between LDL apo-B and total cholesterol (Table 4), the higher apolipoprotein levels in Finnish boys are not surprising. Also, a part of the differences might be explained by differences in laboratory techniques. The observation that there was little variation in the composition of LDL- and HDL-particles in Finnish boys [32] is confirmed in the present study (Table 4). However, data of 5–17 year old white boys from the Bogalusa Heart Study indicated a heterogeneity among HDL-particles [33]. The HDL-cholesterol to apo-A<sub>1</sub> ratio was age-dependent in that study, which might have weakened the correlation between HDL-cholesterol and apo-A<sub>1</sub>.

### *Diet and lipoproteins*

No significant correlations were found between dietary variables and serum lipids or apolipoproteins within our sample. This could be expected because dietary habits in the group were fairly homogeneous, and most of the apparent differences in nutrient intake were prob-

ably due to random within-subject fluctuations rather than to true long-term differences in dietary pattern. The degrading effect on correlations between diet and serum lipids of such intra-individual variation has been discussed extensively [34]. However, the method used is suitable for characterizing the average nutrient intake of the study population, which was our purpose.

In summary, our hypothesis that a typical, olive-oil-rich Cretan diet causes a relatively high HDL-cholesterol to total cholesterol ratio is not supported by the present findings.

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