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Serum cholesteryl ester fatty acids and their relation with serum lipids in elderly men in Crete and The Netherlands

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This study describes dietary fatty acid intake, as assessed from serum cholesteryl ester composition, and its relation to serum lipoprotein levels in 100 age-matched elderly men from Crete and Zutphen. All were survivors of the respective cohorts of the Seven Countries Study [Keys A (1980) *Seven countries: a multivariate analysis of death and coronary heart disease*. Cambridge, MA: Harvard University Press]. A significantly higher percentage of oleic acid and a significantly lower percentage of linoleic acid in the cholesteryl esters was observed in the Cretan compared with the Zutphen men. The mean serum total cholesterol levels of the elderly men in Crete and Zutphen were similar (5.98 and 5.92 mmol/l, respectively), but in the Cretans the HDL cholesterol level was significantly higher (1.28 vs 1.09 mmol/l). The percentage of smokers and the average body mass index did not differ between the Cretan and Zutphen men, but the percentage of severely overweight men was three times higher in the Cretan cohort. Among men in Zutphen the oleic acid content of the cholesteryl esters was positively associated with the HDL cholesterol level, independent of the effects of age, body mass index, cigarette smoking and alcohol intake. However, the difference in HDL cholesterol level between Zutphen and Crete could be explained to only a minor extent by differences in serum cholesteryl oleic acid, smoking, or obesity. These data are compatible with a positive effect of oleic acid intake on serum HDL cholesterol concentrations within elderly men in The Netherlands, but the large difference in apparent oleic acid intake between the Dutch and the Cretan group made only a small contribution towards explaining the observed difference in mean HDL cholesterol levels.

In the Seven Countries Study, the incidence of coronary heart disease (CHD) in the Cretan cohort was lower than expected from the levels of the major risk factors, e.g. serum total cholesterol, cigarette smoking and blood pressure (Keys, 1970, 1980*a*). The typical Cretan diet, which includes a large amount of olive oil, may play a role in explaining the low incidence of CHD (Keys, Aravanis & Sdrin, 1966; Keys *et al.*, 1986). Such a diet would be expected to produce relatively high HDL and low LDL cholesterol levels (Mensink & Katan, 1987, 1989). This could not be confirmed, however, in an

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international study among schoolboys. In spite of their high olive oil consumption, boys in Crete did not have a lower total cholesterol or total to HDL cholesterol ratio than boys in Western Europe and the USA (Aravanis *et al.*, 1988). Within populations HDL cholesterol seems to be a protective factor against CHD (Abbott *et al.*, 1988), although some long-term follow-up studies failed to find such a relationship (Keys, 1980*b*; Keys *et al.*, 1984).

In order to study the epidemiological relations between dietary fatty acids and serum HDL cholesterol levels, we have now compared elderly men from the cohorts of the Seven Countries Study from Crete (Greece) and Zutphen (The Netherlands). The fatty acid composition of serum cholesteryl esters was used as a biochemical indicator of the intake of unsaturated fatty acids.

Methods

From 1960 onwards, cohorts of middle-aged men in Zutphen (The Netherlands) and Crete (Greece) were examined according to a standardized protocol (Keys *et al.*, 1967). In the spring of 1985, 367 of the 555 survivors of the cohort from Zutphen were re-examined. In the spring of 1986, 110 Cretan survivors were also re-examined. The Cretan cohort consists of men from Kastelli, Thrapsano, Xidas, Voni, Apostoli and Agies Pareskies, small villages 25–40 km south-east of Iraklion. Three of the 110 Cretan men did not belong to the cohort established in 1960, and for seven men no medical history was available. These 10 men were excluded. For the remaining 100 Cretan men an age-matched sample was drawn from the 367 survivors in Zutphen.

The investigation included a medical history and a physical examination. Resting electrocardiograms were made, and coded according to the Minnesota Code by one observer at the ECG coding centre at the Leiden Academic Hospital (Rose & Blackburn, 1968). The criteria for myocardial infarction described by Keys were used (Keys, 1970). The prevalence codes 30 (major Q waves) and 31 (lesser Q waves plus major T wave findings) were taken as evidence for definite myocardial infarction. Body height and weight (lightly clothed) were measured according to the protocol of the Seven Countries Study (Keys *et al.*, 1967). Blood pressure was measured twice in supine position at the end of the physical examination. Information about alcohol consumption was obtained by the cross-check dietary history method (den Hartog *et al.*, 1965) in Zutphen, but not in Crete. Information about smoking habits was collected by questionnaire in a standardized interview in both cohorts. Non-fasting blood samples were collected. The sera were stored at −20°C until transport to Wageningen. In Wageningen the sera were stored at −80°C until analysis. Serum total cholesterol, HDL cholesterol and the fatty acid composition of the cholesteryl esters of both cohorts were analysed at the Department of Human Nutrition, Agricultural University Wageningen. Serum total cholesterol was determined enzymatically with a CHOD-PAP monotest kit from Boehringer Mannheim (Siedel *et al.*, 1981), using serum calibrators provided and certified by The Netherlands Foundation for Quality Control of Chemical Analyses in Epidemiological Research (Boerma *et al.*, 1986). The laboratory was certified by the Foundation as meeting the WHO criteria for accuracy and precision. HDL cholesterol was determined enzymatically after precipitation of the apo B-containing lipoproteins with dextran sulphate–Mg^{2+} (Warnick, Benderson & Albers, 1982). Bias for total and HDL cholesterol were also checked using control sera provided by the Centers for Disease Control, Atlanta, Georgia. Mean bias relative to target values was 0.1% for total cholesterol and −3.2% for HDL cholesterol.

For the analysis of the fatty acid composition of the cholesteryl esters, the lipids were extracted with a mixture of isopropanol, water and *n*-octane (Wang & Peter, 1983). The cholesteryl esters were separated from the other lipids by thin layer chromatography, using petroleum ether (bp 40–60°C)-diethylether-acetic acid (160:30:5, by volume) as solvent. The cholesteryl esters were saponified with NaOH/methanol and the fatty acids were methylated with BF_3/methanol (Metcalfe, Schmitz & Pekka, 1966). The fatty acid methyl esters were extracted with petroleum ether (bp 40–60°C) and separated on a gas chromatograph (Packard, model 427) with two glass columns (180 cm × 2 mm) packed with 15% CP Sil 84 on Chromosorb W HP (100–120 mesh). The temperature was programmed from 170 to 210°C and the peaks were quantified with a flame ionization detector and a Spectra Physics 4100 integrator. The coefficient of variation for the major fatty acids 18:1 and 18:2 was 4.7% and 3.2%, respectively.

Statistical analyses were carried out using SPSS-X package of statistical software (Aravanis & Ioanidas, 1986). Means and standard deviations were calculated. Differences between the cohorts and between smokers and non-smokers were tested for statistical significance with Student's *t*-test. Variables that did not follow a Gaussian distribution (diastolic blood pressure and SGPT) were transformed to their natural logarithm. Differences in percentages of smokers, prevalence of myocardial infarction and severe overweight (Quetelet index = weight/height^2 ≥ 30 kg/m^2) between the cohorts were tested by χ^2. Spearman correlation coefficients were computed to test for associations between different variables. Multiple linear regression analyses were carried out with serum total cholesterol and HDL cholesterol as dependent variables. In analyses using the data of both cohorts, cohort was entered as a dummy variable (0 = Zutphen, 1 = Crete). Adjustments for differences in HDL cholesterol between the two cohorts were made with analyses of covariance. A two-sided *P*-value of <0.05 was considered statistically significant.

Results

Some characteristics of the men from Crete and Zutphen are shown in Table 1. HDL cholesterol and systolic blood pressure were significantly higher in Crete than in Zutphen. The average Quetelet index, total cholesterol and diastolic blood pressure did not differ significantly between the two cohorts. The percentage of severely overweight men in Crete was, however, significantly higher than in Zutphen (Table 2). The prevalence of myocardial infarction was about twice as high in Zutphen as in Crete. The average total cholesterol level was similar among smokers and non-smokers in both Crete and Zutphen. In Zutphen the HDL cholesterol level of non-smokers (1.12 mmol/l) was significantly higher than that of smokers (1.00 mmol/l). The HDL cholesterol levels of smokers and non-smokers did not differ significantly in Crete.

The fatty acid composition of the cholesteryl esters is given in Table 3. The percentage of saturated fatty acids (16:0 and 18:0) was slightly but significantly higher among the Zutphen men. The Cretan men had a markedly significantly higher percentage of oleic acid (18:1) and a significantly lower percentage of linoleic acid (18:2). Variation in oleic and linoleic acid was much higher within the Zutphen than within the Cretan group of men. The percentages of

Table 1. Characteristics of 100 age-matched elderly men from Crete and from Zutphen

	Crete		*Zutphen*	
	Mean	*SD*	*Mean*	*SD*
Age (years)	73.3	4.6	73.3	4.6
Quetelet index (kg/m^2)	25.5	3.5	24.8	3.1
Total cholesterol (mmol/l)	5.98	1.08	5.92	0.93
HDL cholesterol (mmol/l)	1.28	0.29	1.09	0.26**
Systolic blood pressure (mmHg)	157.9	22.5	150.6	21.3*
Diastolic blood pressure (mmHg)	85.3	9.8	85.5	11.3

* $P < 0.05$.
** $P < 0.001$.

Table 2. Percentage of prevalent cases with myocardial infarction, cigarette smokers and severely overweight men in Crete and Zutphen

	Crete		*Zutphen*	
	n	%	*n*	%
Myocardial infarction	100	4.0	97	9.3
Present smokers	100	38.0	100	27.0
Quetelet index ≥30 kg/m²	100	10.0	100	3.0*

* $P < 0.05$.

Table 3. Mean fatty acid composition of cholesteryl esters in serum of 92 elderly men from Crete and 97 elderly men from Zutphen[a]

Fatty acid	*% methylesters*		*P-value*
	Crete	*Zutphen*	
16:0	11.1 ± 1.0	11.9 ± 1.3	<0.001
16:1	3.2 ± 1.1	2.9 ± 1.6	0.213
18:0	0.7 ± 0.3	1.1 ± 0.5	<0.001
18:1	31.0 ± 2.7	21.4 ± 3.9	<0.001
18:2 (n-6)	41.9 ± 3.7	53.1 ± 6.5	<0.001
18:3 (n-3)	0.9 ± 0.5	0.3 ± 0.4	<0.001
20:4 (n-6)	6.5 ± 1.6	4.5 ± 1.5	<0.001
Others	4.6 ± 3.3	4.7 ± 3.7	0.891
Ratio 18:2/18:1	1.37 ± 0.20	2.60 ± 0.75	<0.001

[a]Results are expressed as mean % (by weight) methylesters ± SD.

linolenic acid (18:3) and arachidonic acid (20:4) were significantly higher in the Cretan than in the Zutphen men. The association between the percentage of oleic acid in the cholesteryl esters and HDL cholesterol was not significant in Crete, but significantly positive in Zutphen (Table 4). The percentage of linoleic acid was positively associated with HDL cholesterol in Crete. This association was inverse in Zutphen. The Quetelet index was inversely related to HDL cholesterol both in Crete and Zutphen. In Zutphen, but not in Crete, age was correlated positively with HDL cholesterol. Information about alcohol consumption was available only in Zutphen. In this cohort, alcohol consumption was positively associated with HDL cholesterol. This relation was borderline significant ($P = 0.06$).

The linoleic acid content of the cholesteryl esters was inversely related to total cholesterol in Zutphen only (Table 4). Quetelet index was positively associated with total cholesterol, but this association reached statistical significance only in Crete. Age was inversely related to total cholesterol. This association also was significant only in Crete. Combined analyses of Crete and Zutphen confirmed the positive association between Quetelet index and total cholesterol and the inverse relation between age and total cholesterol.

Independent relations between serum cholesteryl ester fatty acids and serum HDL

Table 4. Univariate Spearman rank correlation coefficients between lipid fractions and their determinants for elderly men from Crete and Zutphen separately and together

	Crete		*Zutphen*		*Crete + Zutphen*	
	Chol.	*HDL*	*Chol.*	*HDL*	*Chol.*	*HDL*
18:4 (% esters)	0.01	−0.11	0.18	0.33***	0.09	0.40***
18:2 (% esters)	−0.01	0.28**	−0.23*	−0.24*	−0.11	−0.33***
QI (kg/m²)[a]	0.20*	−0.24*	0.15	−0.31**	0.19**	−0.22**
Age (years)	−0.27***	−0.14	−0.11	0.21*	−0.19**	0.03
Alcohol (g/day)			−0.01	0.19		

[a] Quetelet index.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

and total cholesterol were investigated by multiple regression. In Zutphen the percentage of oleic acid in the cholesteryl esters was significantly associated with HDL cholesterol, independent of Quetelet index, cigarette smoking and alcohol consumption (Table 5). In Crete only Quetelet index was significantly related to HDL cholesterol. In the combined analyses, Quetelet index, cigarette smoking and cohort (Crete or Zutphen) contributed significantly to HDL cholesterol. Adjustment for Quetelet-index, cigarette smoking and the percentage of oleic acid in the cholesteryl esters produced only a minor reduction in the difference in mean HDL cholesterol level between the Zutphen and Cretan cohort (adjusted HDL cholesterol levels in Zutphen and Crete were 1.11 and 1.26 mmol/l, respectively). Only age was independently related to total cholesterol in Crete and in the combined analyses of the two cohorts (Table 6). The inverse relation between the linoleic acid content of the cholesteryl esters and total cholesterol was borderline significant (P = 0.05).

Table 5. Multivariate analysis of relations between HDL cholesterol and its determinants in elderly men from Crete and Zutphen

Determinant	*HDL cholesterol (mmol/l)*					
	Crete		*Zutphen*		*Crete + Zutphen*	
	B	*SE(B)*	*B*	*SE(B)*	*B*	*SE(B)*
18:1 (% esters)	−0.011	0.011	0.018	0.006**	0.009	0.006
Quetelet index (kg/m²)	−0.022	0.009*	−0.028	0.007***	−0.024	0.006***
Cigarette smoking (0, 1)	−0.102	0.065	−0.122	0.050*	−0.113	0.040**
Alcohol (g/day)			0.003	0.001*		
Cohort[a]					0.149	0.066*
R^2 (%)	7.5		32.7		24.2	

[a] 0 = Zutphen, 1 = Crete.
B = multiple regression coefficient.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 6. Multivariate analysis of relations between total cholesterol and its determinants in elderly men from Crete and Zutphen

Determinant	*Total cholesterol (mmol/l)*					
	Crete		*Zutphen*		*Crete + Zutphen*	
	B	*SE(B)*	*B*	*SE(B)*	*B*	*SE(B)*
18:2 (% esters)	−0.011	0.030	−0.028	0.014	−0.025	0.013
Quetelet index (kg/m²)	−0.032	0.032*	0.044	0.032	0.036	0.022
Age (years)	−0.056	0.024*	−0.024	0.021	−0.040	0.016
Cohort[a]					−0.181	0.207
R^2 (%)	7.5		6.9		6.8	

[a] 0 = Zutphen, 1 = Crete.
B = multiple regression coefficient.
* $P < 0.05$.

Discussion

The fatty acid composition of serum cholesteryl esters can be used as an indicator for the fatty acid composition of the diet (Dyerberg, Bang and Hjorne, 1975; Dayton *et al.*, 1966; Moilanen *et al.*, 1983; Nikkari *et al.*, 1983*a*). In Crete, the percentage of oleic acid in the cholesteryl esters was much higher than in Zutphen. This high percentage of oleic acid reflects a high olive oil consumption. On the other hand, the percentage of linoleic acid in Zutphen was higher than in Crete, reflecting a higher linoleic acid intake in Zutphen. The proportions of C18:3(n−3) (α-linolenic acid) and C20:4(n−6) (arachidonic acid) were higher in Cretan than in Dutch men (Table 3). However, duplicate diet analysis showed the consumption of α-linolenic and arachidonic acid to be lower in Crete than in Zutphen (Kromhout *et al.*, unpublished). Possibly the LCAT enzyme, which synthesizes cholesteryl esters from cholesterol and phospholipid, incorporated higher levels of these two polyunsaturated fatty acids into cholesteryl esters in Cretan men because of a limited supply of the preferred fatty acid, linoleic acid.

The Cretan men had significantly higher average HDL cholesterol levels than the Zutphen men. However, only a minor part of this difference could be explained by the difference in the percentage of oleic acid in serum cholesteryl esters. Adjustment for the observed differences in oleic acid plus obesity plus cigarette smoking between the Cretan and the Dutch men left 0.15 out of the original difference of 0.19 mmol/l in HDL cholesterol still unaccounted for (Table 5). A prime candidate for explaining the higher HDL levels in the Cretan men is alcohol intake, which at least in the 1960s was appreciably higher in men in Crete than in Zutphen (Kromhout *et al.*, 1989). Unfortunately, alcohol intake was not measured in Crete in the present study.

Among men in Zutphen, but not in Crete, the percentage of oleic acid in the cholesteryl esters was positively associated with HDL cholesterol, independent of known determinants such as smoking, alcohol intake and obesity. The percentage of linoleic acid in the cholesteryl esters was inversely related to HDL cholesterol in univariate, but not in multivariate analyses. This may be due to the strong inverse correlation ($r = -0.73$, $P < 0.001$) between the percentages of oleic acid and linoleic acid in the cholesteryl esters. The fatty acids in the cholesteryl esters are expressed as a percentage of the total fatty acid content, and oleic and linoleic acid are the dominating fatty acids in the cholesteryl esters. This means that a low percentage of oleic acid goes together with a high percentage of linoleic acid and vice versa. It is therefore impossible to say which of these two fatty acids is most important in relation to HDL cholesterol. A low percentage of linoleic acid in the cholesteryl esters within populations is a risk factor for CHD (Kingsbury *et al.*, 1969*a,b*; Nikkari *et al.*, 1983*b*; Moilanen *et al.*, 1985). In the Cretan cohort the percentage of linoleic acid is rather low. Greenland Eskimos, a population with just as low an incidence of CHD as the Cretans, have a very low percentage of linoleic acid in their cholesteryl esters (Dyerberg, Bang & Hjorne, 1975). In a cross-cultural study of 7–8-year-old boys, Knuiman *et al.* (1980) found that both the linoleic acid content of cholesteryl esters and the serum cholesterol level were higher in affluent populations with a high fat intake than in populations in Africa and Asia with a low fat intake and a low rate of CHD.

These results suggest that the percentage of linoleic acid in the cholesteryl esters is not an important determinant of differences in serum cholesterol and CHD incidence between populations. Evidently the large differences in saturated fat intake override the effect of linoleic acid in comparisons between populations.

In Zutphen, but not in Crete, an inverse association was observed between the linoleic acid content of the cholesteryl esters and total cholesterol. This inverse association was also found in children (Moilanen *et al.*, 1986) and is in agreement with the fact that polyunsaturated fatty acids decrease total cholesterol (Keys, Anderson & Grande, 1965; Becker *et al.*, 1983). The reason that no association was found between the percentage of linoleic acid of the cholesteryl esters and total cholesterol within the Cretan cohort may be due to the smaller variation in this fatty acid in Crete. The range for 95% of the values was 34.5–49.3 in Crete and 40.1–66.1 in Zutphen.

The results of the present study suggest that oleic acid intake as reflected by the fatty acid composition of the serum cholesteryl esters may have an effect on HDL cholesterol levels in elderly Dutch men independent of known determinants such as alcohol intake, smoking and obesity. However, the large difference in oleic acid intake between the Cretan and the Dutch cohort could explain only a minor part of the difference in HDL between the two groups; other, unmeasured factors must be responsible for this difference, with alcohol intake being one likely candidate.

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