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Serum plant sterols as surrogate markers of dietary compliance in familial dyslipidemias

Rocío Mateo-Gallego, Lucía Baila-Rueda, Theodora Mouratidou, Ana M. Bea, Sofía Perez-Calahorra, Ana Cenarro, Luis A. Moreno, Fernando Civeira

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Authors: Rocío Mateo-Gallego[1], Lucía Baila-Rueda[1], Theodora Mouratidou[2], Ana M. Bea[1], Sofía Perez-Calahorra[1], Ana Cenarro[1], Luis A. Moreno[2], Fernando Civeira[1].

Centers: [1]Unidad de Lípidos and Laboratorio de Investigación Molecular, Hospital Universitario Miguel Servet, Instituto Aragonés de Ciencias de la Salud (I+CS), Zaragoza, Spain. [2]GENUD (Growth, Exercise, Nutrition and Development) Research Group, Faculty of Health Sciences, University of Zaragoza, C/ Domingo Miral s/n, 50009 Zaragoza, Spain.

Address for correspondence: Rocío Mateo-Gallego. Unidad de Lípidos. Hospital Universitario Miguel Servet. C/ Padre Arrupe, s/n, 50009, Zaragoza, Spain.

Phone: +34 976765500. Fax: +34 976369985. E-mail: rmateo.iacs@aragon.es

Abbreviations

CVD; Cardiovascular disease

ADH; Autosomal dominant hypercholesterolemia

FCHL; Familial combined hyperlipidemia

TC; Total cholesterol

BMI; Body mass index

CRP; C-reactive protein

FFQ; Food frequency questionnaire

PCA; Principal component analysis

GGT; γ-glutamyl transpeptidase

ABSTRACT

Background and Aims: A well-balanced diet is the first line treatment in hyperlipidemia. The objective was to study the association between serum phytosterols and dietary patterns to use them as surrogate markers of dietary compliance in primary dyslipidemias.

Methods: 288 patients with primary hyperlipidemias (192 autosomal dominant hypercholesterolemia (ADH) and 96 familial combined hyperlipidemia (FCHL)) were included. Principal factor analysis identified 2 major dietary patterns using a 137-item food frequency questionnaire. “Vegetable & Fruits pattern” was characterized by higher intake of of fruits, green beans, nuts, tomatoes, roasted or boiled potatoes, lettuce and chard and lower of processed baked goods, pizza and beer. “Western pattern” was positively characterized by hamburgers, pasta, sunflower oil, rice, chickpeas, whole milk, veal, red beans and negatively with white fish. Serum non-cholesterol sterols were determined by HPLC-MS/MS.

Results: Plant sterols to-total cholesterol (TC) levels were lower with a higher adherence to a “Vegetable & Fruits pattern” ($P = 0.009$), mainly in ADH subjects ($R^2 = 0.019$). Their concentration was greater with higher compliance to “Western pattern” especially in FCHL ($P = 0.014$). Higher levels of synthesis markers to TC with a greater adherence to “Vegetable & Fruits pattern” was found ($P = 0.001$) ($R^2 = 0.033$ and $R^2 = 0.109$ in ADH and FCHL respectively).

Conclusion: In subjects with primary dislipidemia, dietary patterns associate with serum absorption and synthesis markers, but no with lipid concentrations. The influence of diet on non-cholesterol sterols levels is not powerful enough to use them as subrogate markers.

KEYWORDS

Plant sterols, phytosterols, familial hyperlipidemias, dietary patterns, dietary compliance.

INTRODUCTION

The strong relationship between plasma cholesterol and cardiovascular disease (CVD), the leading cause of mortality in the world, is well accepted [1,2]. Lifestyle behavioural changes, including dietary modifications, increased physical activity and weight loss in overweight or obese patients, are the first-line option treatment in hypercholesterolemias. This is also reflected in the National Cholesterol Education Program (NCEP) dietary guidelines for the prevention of CVD recommending a healthy dietary pattern rich in monounsaturated fatty acids provided by plant sources, fruits, vegetables, whole grains, and low-fat dairy products and low in saturated fatty acids and cholesterol [3].

Dietary assessment is important in the following-up of the patients so as to identify "unhealthy" dietary habits to promote adherence to healthier dietary patterns. Dietary questionnaires such as food frequency questionnaires, 24 hour recalls or food records are commonly used to assess dietary intake, however, such methods come with limitations as reliance on memory, misreporting and should be considered when evaluating nutrition behaviours [4,5]. Subrogate parameters which could objectively evaluate dietary compliance would be very useful both in epidemiological studies and in clinical practice.

Serum levels of phytosterols, commonly known as plant sterols, and cholestanol are positively correlated with cholesterol absorption and their ratios to cholesterol (relative concentrations) are considered to be reliable markers of intestinal sterols absorption efficiency [6,7]. Serum phytosterols are only partially dependent to their amount in the diet although this association has not been studied in subjects with primary dyslipidemias who show an abnormal cholesterol homeostasis [8,9].

Dietary pattern analysis has emerged as an alternative approach to examine the relationship between diet and the risk of chronic diseases; conceptually, dietary patterns provide a broader picture of food and nutrient consumption, and may thus be more predictive

of disease risk than individual foods or nutrients [10,11]. The aim of the study was to examine the association between serum phytosterols levels and dietary patterns in primary dyslipidemias by proposing them as surrogate markers of dietary compliance in patients with primary dyslipidemias.

MATERIALS AND METHODS

Study population

Patients attending to the Lipid Unit of the Hospital Universitario Miguel Servet (Zaragoza, Spain) from January 2011 to September 2012 were recruited. 288 subjects with familial hypercholesterolemias were recruited as part of a genetic and metabolic wider study whose study details has been already published elsewhere [12]. Inclusion criteria were being over 18 years of age and the presence of familial hyperlipidemia by including autosomal dominant hypercholesterolemia (ADH) and familial combined hyperlipidemia (FCHL). ADH was diagnosed in subjects with off-treatment LDL cholesterol levels above the age- and sex-specific 95^{th} percentile of a Spanish reference population, triglyceride below 200 mg/dL and familial vertical transmission with at least one first-degree relative with LDL cholesterol above age- and sex-specific 95^{th} percentiles. The diagnosis of FCHL was based on the presence of primary combined hyperlipidaemia in untreated patients whose serum cholesterol and triglyceride concentrations were above the sex- and age-specific 90th percentiles for the Spanish population, serum total apolipoprotein B levels $\geq$ 120 mg/dL and there was at least one first-degree relative with hyperlipidemia (total cholesterol (TC) and/or triglycerides >90th percentile) [13]. Secondary causes of hyperlipidaemia (e.g. body mass index (BMI) $\geq$ 30 kg/m^2, alcohol intake over 30 gr. and 20 gr. in men and women respectively) and subjects with plant sterols supplements intake were excluded. Written informed consent was obtained

by all study participants. The study protocol was approved by the Ethical Committee of our Institution (Comité Ético de Investigación Clínica de Aragón).

Clinical and laboratory determination

Clinical parameters obtained included anthropometric measures (weight, height and waist circumference) and blood pressure. BMI was calculated (weight in kg. divided by the square of height in meters) and all subjects were assessed for personal and/or family history of early-onset coronary heart disease, clinical history, tobacco consumption and demographic characteristics by a personal interview.

Fasting blood was drawn following at least 4 weeks without lipid-lowering drugs treatment. Cholesterol, triglycerides, HDL cholesterol and γ-glutamyl transpeptidase (GGT) were measured by spectrophotometry with standard enzymatic methods. LDL cholesterol was estimated with the Friedewald formula when serum triglycerides were <400 mg/dL. Non-HDL cholesterol was calculated as TC minus HDL cholesterol. Apolipoprotein B, lipoprotein(a), and C-reactive protein (CRP) were determined by nephelometry using IMMAGE-Immunochemistry System (Beckman Coulter).

Dietary assessment

Dietary intakes were determined using an interviewer-administered 137-item food frequency questionnaire (FFQ). One registered dietician (RM-G) performed the interviews. More details of the FFQ validity, which has been previously used to study other diet-disease association including plant sterols, could be found elsewhere [14-16]. Food and nutrient intakes were calculated as frequency x nutrient composition of specified portion sizes, where frequencies were measured in 9 categories (never, 1-3 times a month, 1 time a week, 2-4 times a week, 5-6 times a week, 1 time a day, 2-3 times a day, 4-6 times a day and > 6 times a day) for each food item. The total energy and nutrients intakes were calculated based on previously validated Spanish food composition tables [17]. When possible and applicable, the 137 foods

were grouped into categories based on similar nutritional values. Food items (N = 18) with low prevalence of consumption (less than 15%) were not considered in the final analysis (N = 119) to avoid possible bias in the dietary patterns calculation.

Serum non-cholesterol sterols determination

Serum non-cholesterol sterol concentrations were analysed by high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) [18]. Briefly, ([$^2H_6$] cholesterol-26,26,26,27,27,27 D6) (4 µg/g) was added to serum (0.1 ml) as the internal standard. After alkaline hydrolysis, extraction and solid phase extraction, the sterols were separated using reverse-phase C18 HPLC. A 40 µl aliquot of the extract (100% 2-propanol) was loaded onto a RP-HPLC column (Zorbax Eclipse Plus C_{18} 2.1 x 150 mm, 3,5 µm particle; Agilent, Spain) equipped with a guard column (C_{18}, 4 x 2,5 mm). The HPLC (Agilent 1200RRLC) was coupled to a 4000 QTrap triple quadrupole ion trap mass spectrometer (Applied Biosystems, Foster City, CA) through an APCI by Heated Nebulizer (Turbo VTM Source). In each run, cholestanol, campesterol, sitosterol, sitostanol and stigmasterol were quantified.

Statistical analysis

Statistical analysis was performed using SPSS software version 15.0 (Chicago, Illinois, USA) using a significance level of $P < 0.05$.

Data are expressed as means ± standard deviation (SD) for continuous variables with normal distribution and medians (percentile 25 – percentile 75) for variables with a skewed distribution. Student-t or Mann-Whitney tests were used accordingly. Categorical variables were compared using a chi-square test. ANOVA and Kruskal-Wallis tests were performed to multiple independent variables comparison. Non-cholesterol sterols levels were adjusted by those variables which have been shown more influential in its concentration: age, gender, BMI and APOE genotype [19]. PCA with varimax rotation was used to derive dietary patterns based on the 61 foods or food groups [10,20,21]. The factors were rotated by an orthogonal

transformation (resulting in uncorrelated factors) to achieve a simpler structure with greater interpretability. In determining the number of factors to retain, we considered components with an eigenvalue >1, the Scree test and the interpretability of the factors. The factor score for each pattern was constructed by summing observed intakes of the component food items weighted by factor loadings so each subject had a score for each dietary pattern with a higher score indicating higher adherence to the respective pattern [22]. Factor loadings quintiles were calculated for each different dietary pattern to study the association with non-cholesterol sterols levels.

RESULTS

The study group was composed of 288 subjects (48% men), of whom 192 were diagnosed with ADH and 96 subjects with FCHL. **Table 1** presents the main clinical and biochemical characteristics by dyslipidemia type. The FCHL group had a higher percentage of men (65.6% vs. 39.8%, $P = < 0.001$), smokers (35.4% vs. 22.5% in FCHL and ADH respectively, $P = 0.048$), had higher BMI (26.3±2.12 vs. 24.3±2.79 in FCHL and ADH respectively, $P < 0.001$) and waist circumference (93.2±8.14 vs. 84.0±10.5 cm. in FCHL and ADH respectively, $P < 0.001$) than AHD group. Regarding biochemical parameters, patients diagnosed with ADH, as expected, showed higher levels of LDL cholesterol (219 (198-250) vs. 204 (181-228) mg/dL in ADH and FCHL respectively, $P < 0.001$), HDL cholesterol (56.0 (45.3-68.0) vs. 41.5 (36.3-52.8) mg/dL in ADH and FCHL respectively, $P < 0.001$) and apolipoprotein A1 (158±35.1 vs. 143±28.1 mg/dL in ADH and FCHL respectively, $P < 0.001$) and lower values of triglycerides (95.5 (77.0-131) vs. 246 (194-382) mg/dL in ADH and FCHL respectively, $P < 0.001$). Adjusted non-cholesterol sterols concentrations and their ratios to TC by type of dyslipidemia are shown in **Table Supplementary 1**. In general, the ADH group had higher levels of non-cholesterol sterols which have been established as absorption markers such as

cholestanol, desmosterol and campesterol especially with their ratios to TC. FCHL group showed higher concentrations of cholesterol synthesis markers particularly of lanosterol concentration.

Factor analysis (principal components) to derive dietary patterns based on the 61 foods or food groups were performed and 1 major and 3 minor patterns (accounting for 18.8% of the variance) were identified. Thus, we extracted 2 factors in the final model whose factor-loading matrixes are presented in **Table 2**. The larger the loading of a given food item to the factor, the greater the contribution of that food item to a specific factor. The first factor was positively correlated with consumption of oranges, bananas, apples, green beans, nuts, tomatoes, roasted or boiled potatoes, lettuce and chard whilst negatively with processed baked goods, pizza and beer. The second factor was loaded positively with hamburgers, pasta, sunflower oil, rice, chickpeas, whole milk, veal, red beans and negatively with white fish. Components were named as "Vegetable & Fruits dietary pattern" and the second as "Western dietary pattern", which explained 6.85% and 4.12% of the total diet variance respectively. The two other minor patterns that were identified did not appear to represent a clear dietary pattern and analyses did not suggest a significant association between these patterns and non-cholesterol sterols (data not shown).

Subjects characteristics across quintiles of dietary patterns ("Vegetable & Fruits dietary pattern" and "Western dietary pattern") scores are described in **Table Supplementary 2** and **Table Supplementary 3** respectively. Those subjects with a higher score for the "Vegetable & Fruits dietary pattern" were more likely to be older, have a higher intake of energy, total fat, phytosterols, fiber and lower carbohydrates consumption. The percentage of men was higher in the lowest quintile although a tendency across quintiles was not observed. Regarding "Western dietary pattern", those subjects with a higher adherence were younger and presenting an upper intake of energy, carbohydrates, cholesterol and lower of

monounsaturated fat consumption. Significant differences were found across quintiles in protein and phytosterols intake although a clear tendency was not found. A higher percentage of smokers was found in the highest quintile of "Western dietary pattern" compliance.

The association of non-cholesterol sterols with dietary patterns is described in **Table 3** and **4** ("Vegetable & Fruits dietary pattern" and "Western dietary pattern" respectively). Those subjects with a higher adherence to the "Vegetable & Fruits dietary pattern" showed lower levels of adjusted absorption (phytosterols-to TC) markers compared to those with lower adherence (2.59 (2.44-2.88) x 10^{-2} mg/dL in Q1 versus 2.48 (2.30-2.61) x 10^{-2} mg/dL in Q5, $P = 0.009$ in all subjects). This was mainly noted in stigmasterol-to-TC (0.42 (0.36-0.43) x 10^{-2} mg/dL in Q1 versus 0.40 (0.34-0.42) x 10^{-2} mg/dL in Q5, $P = 0.002$ in all subjects) and sitosterol-to-TC (1.65 (1.57-1.87) x 10^{-2} mg/dL in Q1 versus 1.60 (1.51-1.68) x 10^{-2} mg/dL in Q5, $P = 0.044$ in all subjects). This tendency was mainly observed in subjects diagnosed with ADH. Cholestanol-to-TC levels did not show a clear tendency across quintiles, which was not statistically significant in any of both dyslipidemias. The concentration of adjusted synthesis-to-TC (lanosterol and desmosterol) increased across "Vegetable & Fruits dietary pattern" score quintiles which was observed both in ADH and in FCHL (0.70 (0.67-0.75) x 10^{-2} mg/dL in Q1 versus 0.75 (0.71-0.80) x 10^{-2} mg/dL in Q5, $P = 0.001$ in all subjects). Regarding "Western dietary pattern", those subjects with a higher adherence were likely to have higher levels of adjusted absorption (phytosterols-to-TC) markers compared to those in the lowest (2.39 (2.27-2.55) x 10^{-2} mg/dL in Q1 versus 2.54 (2.40-2.71) x 10^{-2} mg/dL in Q5, $P = 0.012$ in all subjects) although the trend was not fully clear. As in the first dietary pattern, this effect was mainly present in campesterol-to-TC (0.39 (0.36-0.41) x 10^{-2} mg/dL in Q1 versus 0.40 (0.38-0.46) x 10^{-2} mg/dL in Q5, $P = 0.042$ in all subjects), stigmasterol-to-TC (0.36 (0.35-0.41) x 10^{-2} mg/dL in Q1 versus 0.41 (0.35-0.42) x 10^{-2} mg/dL in Q5, $P = 0.022$ in all subjects) and sitosterol-to-TC levels (1.56 (1.49-1.66) x 10^{-2} mg/dL in Q1 versus 1.62 (1.51-

1.81) x 10^{-2} mg/dL in Q5, $P = 0.037$ in all subjects). FCHL group had more significant differences among quintiles, mainly comparing the highest quintile vs. the lowest one, but the trend was unclear. The adjusted levels of the synthesis markers levels were likely to be lower in those subjects with higher scores for "Western dietary pattern" but no significant differences were found.

Regression analysis showed that "Vegetable & Fruits dietary pattern" adherence was significantly associated to absorption markers, independently of age, BMI, gender and *APOE* genotype, only in ADH subjects (standarized B = -0.156; $P = 0.030$) by determining a 1.9% of the variance. The compliance to this pattern was independently associated to synthesis subrogate markers in ADH and FCHL (standarized B = 0.195; $P = 0.007$ and standarized B = 0.345; $P = 0.001$ respectively) by determining a 3.3% and 10.9% of levels variance in each case. No significant influence of "Western dietary pattern" compliance in non-cholesterol sterols was founded.

DISCUSSION

The results derived from the present study reveal that there is an influence of diet on non-cholesterol sterols levels although the association is not strong enough to consider them as potential subrogate markers of healthy diet compliance. These data agree with previous studies which have stated the diet influence on serum plant sterols levels concentration, not only with phytosterols intake but with other nutrients too, such as the ratio of polyunsaturated/saturated fatty acids [6]. However, the influence of regular dietary phytosterols intake on circulating plant sterols and in plant sterol-to-TC ratios has been estimated in 4.03% and 3.59% respectively in general population but no data were available for subjects with familial dyslipidemias [19]. Otherwise, the high intake of phytosterols, as supplements, is associated with a marked increase in serum plant sterols concentration

[19,23]. According to our data, healthy diet ("Vegetable & Fruits dietary pattern" with a moderate-high intake of plant sterols) compliance independently influence non-cholesterol sterols, mainly in ADH. These levels variance explained by the dietary adherence is around 1.9% in absorption subrogate markers and 3.3% and 10.9% (ADH and FCHL respectively) in synthesis subrogate markers. Despite having analysed dietary patterns beyond of isolated nutrients, the influence is not strong enough and their usefulness as subrogate markers of dietary adherence does not seem to be useful.

A clear association of lipid parameters and a healthy dietary adherence was not found in any of both dyslipidemias. Thus, in non obese subjects with primary dyslipidemias, the effect of diet on lipid metabolism seems to be low, in contrast with the substantial improvement of lipid profile with weigh loss and dietary habits enhancement in obese subjects with FCHL that has been previously proved [3,24]. Given that non-cholesterol sterols, both absorption and synthesis markers, are not markedly influenced by diet in non obese subjects with primary dyslipidemias, they could be more useful for the study of the pathogenic mechanism of the lipoprotein disorder, as previously proposed [25].

The inverse association of cholesterol absorption with synthesis has been well established in general population and our data confirm this relationship also in subjects with primary dyslipidemias. An increase in cholesterol synthesis by the liver occurs when intestinal cholesterol absorption diminishes due to an increase in phytosterols intake [7,26]. An unexpected finding in our study was that those subjects with a high adherence to a healthy diet, rich in vegetables, fruits and phytosterols, had lower plasma plant sterols levels than those subjects with a lower adherence. At the same time, an increase in cholesterol synthesis markers was also observed with a higher adherence which was especially evident in ADH subjects. We hypothesize that a compensatory mechanism could be the responsible for the reduction of serum plant sterols levels when an increase of a healthy diet ("Vegetable & Fruits

dietary pattern") adherence. It maybe possible that in those patients following a healthy diet the excretion of phytosterols to the bile would be increased by compensating the augmentation of those in the serum whereas the cholesterol neosynthesis in the liver would raise, as previously described by Krawczyc M. et al. in gallstone disease [27]. Because of this compensatory mechanism, no differences in the cholesterol levels across quintiles of dietary adherence are observed despite of a better diet and higher intake of phytosterols. These data reinforces the primary cause of the hypercholesterolemia in the studied subjects and explains the poor response to diet modification in some genetic hypercholesterolemias as previously reported [28,29]. Most of our ADH subjects had familial hypercholesterolemia with a pathogenic mutation in the LDLR, a group of subjects with a very limited response to a lipid lowering diet due to the mechanism of their disease. In contrast, the lipid profile of subjects with FCHL is highly dependent of environmental factors, especially to weight gain. However, cholesterol synthesis, the main pathogenic factor in FCHL, is poorly modified by diet in absence of weight loss, in contrast with triglycerides synthesis [30].

The study has several limitations such as the assessment of food intake based on subjective self-reports although we have not studied nutrients or foods by separate but dietary patterns which represent better and more realistic the overall diet and represent a broader picture of dietary habits which constitute strength of the present study. Subjective decisions on the number of patterns to be extracted were made based on empirical guidelines rather than on an exact quantitative solution and this should be considered as another study limitation.

In conclusion, the influence of diet on non-cholesterol sterols concentration is neither completely clear nor sufficiently powerful and a clear tendency was not observed in patients with primary dyslipidemias. Thus, the usefulness of serum plant sterols levels as subrogate makers of dietary adherence have to be reconsidered.

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STATEMENT OF AUTHORSHIP

The author's responsabilities were as follows – RMG: conducted research, collected and analyzed data and performed statistical analysis, wrote the paper and had primary responsability for its final content; LBR: conducted research, collected and analyzed data; TM: performed statistical analysis and provided significant advice; AMB, SPC and AC: conducted research and provided significant consultation; LAM: provided significant advice; FC: conducted research, analyzed data, performed statistical analysis, wrote the paper, provided significant advice and consultation and had primary responsability for its final content.

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CONFLICT OF INTEREST STATEMENT

None.

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Table 1. Factor loading matrix for the two major dietary patterns identified.[a]

	"Vegetable & fruit dietary pattern"	"Western dietary pattern"
Oranges	0.755	
Bananas	0.683	
Apples	0.651	
Green beans	0.428	
Nuts	0.426	
Tomatoes	0.416	
Roasted or boiled potatoes	0.367	
Lettuce	0.330	
Chard	0.305	
Processed baked goods	- 0.301	
Pizza	- 0.395	
Hamburguers		0.526
Pasta		0.518
Sunflower oil		0.515
Rice		0.488
Chickpeas		0.460
Whole milk		0.366
Veal		0.319
White fish		- 0.334
Beer	- 0.327	
Red beans		0.329

[a]Absolute values < 0.30 were not listed in the table for simplicity. Food items with factor loadings < 0.30 for any factor were excluded.

Table 2. Clinical and biochemical characteristics of subjects according to clinical diagnosis. [a]

	ADH N= 192	FCH N = 96	*p*
Males, n (%)	76 (39.8)	63 (65.6)	<0.001
Age, years	44.2±12.3	46.0±11.3	0.208
Tobacco consumption, n (%)			0.048
Smoker	43 (22.5)	34 (35.4)	
Former smoker	53 (27.7)	26 (27.1)	
Non smoker	95 (49.7)	36 (37.5)	
Diabetes, n (%)	0 (0)	1 (1)	0.333
Hypertension, n (%)	27 (14.1)	12 (12.5)	0.433
Systolic blood pressure, mm Hg	127 (119-135)	131 (123-141)	<0.001
Diastolic blood pressure, mm Hg	80.0 (70.0-85.0)	82.5 (77.3-90.0)	<0.001
Body mass index, kg/m^2	24.3±2.79	26.3±2.12	<0.001
Waist circumference, cm	84.0±10.5	93.2±8.14	<0.001
Metabolic syndrome, n (%)	22 (11.5)	50 (52.1)	< 0.001
Total cholesterol, mg/dL	309±48.3	299±44.1	0.106
HDL cholesterol, mg/dL	56.0 (45.3-68.0)	41.5 (36.3-52.8)	<0.001
Non HDL cholesterol, mg/dL	239 (218-271)	246 (222-276)	0.252
LDL cholesterol, mg/dL	219 (198-250)	204 (181-228)	<0.001
Triglycerides, mg/dL	95.5 (77.0-131)	246 (194-382)	<0.001
Apolipoprotein A1, mg/dL	158±35.1	143±28.1	<0.001
Apolipoprotein B, mg/dL	161±33.7	168±31.6	0.104
Lipoprotein(a), mg/dL	27.9 (13.6-59.2)	24.3 (7.60-59.0)	0.193
C reactive Protein, mg/L	1.50 (0.60-3.40)	2.35 (1.10-3.63)	0.009
GGT, IU/L	19.5 (15.0-29.0)	30.0 (19.0-46.0)	<0.001
Glucose, mg/dL	87.8±10.2	92.9±11.1	<0.001
HbA1c, %	5.20 (5.00-5.40)	5.30 (5.10-5.50)	0.033

[a] Values are mean ± standard deviation or median (percentile 25-percentile 75) as applicable.

Table 3. Adjusted non-cholesterol sterols-to-total cholesterol levels among quintiles of "Vegetable & Fruits dietary pattern" scores.[a]

		Cholestanol-to-TC	Stigmasterol-to-TC	Campesterol-to-TC	Sitosterol-to-TC	Synthesis markers to-TC[b]	Phytosterols-to-TC[c]
ADH N = 192	Q1	0.60 (0.56-0.63)	0.42 (0.40-0.43)	0.41 (0.38-0.46)	1.68 (1.61-1.90)	0.71 (0.66-0.74)	2.62 (2.50-2.90)
	Q2	0.61 (0.58-0.64)	0.36 (0.35-0.41)	0.40 (0.38-0.45)	1.58 (1.48-1.68)	0.70 (0.65-0.76)	2.45 (2.29-2.63)
	Q3	0.62 (0.58-0.65)	0.36 (0.35-0.42)	0.40 (0.38-0.45)	1.57 (1.50-1.74)	0.70 (0.66-0.75)	2.44 (2.29-2.70)
	Q4	0.62 (0.58-0.67)	0.40 (0.35-0.42)	0.40 (0.36-0.45)	1.60 (1.53-1.79)	0.72 (0.66-0.78)	2.48 (2.34-2.73)
	Q5	0.60 (0.87-0.65)	0.40 (0.34-0.42)	0.39 (0.36-0.41)	1.60 (1.54-1.68)	0.75 (0.70-0.79)	2.48 (2.31-2.61)
	p	0.561	0.005	0.087	0.009	0.031	0.009
FCHL N = 96	Q1	0.57 (0.53-0.60)	0.42 (0.35-0.52)	0.38 (0.35-0.41)	1.60 (1.47-1.87)	0.70 (0.68-0.75)	2.52 (2.26-2.87)
	Q2	0.58 (0.56-0.61)	0.41 (0.35-0.42)	0.36 (0.35-0.40)	1.60 (1.49-1.65)	0.74 (0.69-0.77)	2.51 (2.30-2.54)
	Q3	0.59 (0.57-0.61)	0.36 (0.35-0.41)	0.39 (0.35-0.40)	1.53 (1.41-1.64)	0.73 (0.69-0.77)	2.29 (2.19-2.56)
	Q4	0.58 (0.55-0.63)	0.41 (0.38-0.47)	0.37 (0.34-0.41)	1.62 (1.56-1.82)	0.77 (0.72-0.78)	2.56 (2.38-2.81)
	Q5	0.58 (0.55-0.59)	0.40 (0.35-0.43)	0.36 (0.34-0.38)	1.61 (1.44-1.69)	0.77 (0.72-0.79)	2.45 (2.23-2.66)
	p	0.303	0.220	0.301	0.441	0.018	0.252
All subjects N = 288	Q1	0.58 (0.54-0.61)	0.42 (0.36-0.43)	0.40 (0.37-0.45)	1.65 (1.57-1.87)	0.70 (0.67-0.75)	2.59 (2.44-2.88)
	Q2	0.61 (0.58-0.63)	0.40 (0.35-0.41)	0.39 (0.36-0.43)	1.59 (1.49-1.66)	0.71 (0.66-0.77)	2.47 (2.30-2.57)
	Q3	0.61 (0.58-0.64)	0.36 (0.35-0.42)	0.40 (0.38-0.44)	1.57 (1.48-1.65)	0.71 (0.67-0.75)	2.44 (2.27-2.58)
	Q4	0.62 (0.57-0.65)	0.40 (0.35-0.42)	0.40 (0.36-0.44)	1.61 (1.53-1.80)	0.73 (0.67-0.78)	2.49 (2.35-2.77)
	Q5	0.59 (0.57-0.62)	0.40 (0.34-0.42)	0.38 (0.35-0.41)	1.60 (1.51-1.68)	0.75 (0.71-0.80)	2.48 (2.30-2.61)
	p	0.044	0.003	0.024	0.012	0.001	0.009

[a]Data (10^{-2}) are expressed as median (percentile 25-pertencil 75). Non-cholesterol sterols-to-total cholesterol levels are adjusted by gender, age, BMI and APOE genotype. *P* refers to statistical differences among quintiles.

[b]Synthesis markers are the sum of lanosterol and desmosterol levels.

[c]Phytosterols are the sum of campesterol, sitosterol, sitostanol and stigmasterol levels.

Table 4. Non-cholesterol sterols-to-total cholesterol levels among quintiles of "Western dietary pattern" scores.[a]

		Cholestanol-to-TC	Stigmasterol-to-TC	Campesterol-to-TC	Sitosterol-to-TC	Synthesis markers-to-TC[b]	Phytosterols-to-TC[c]
ADH N = 192	Q1	0.62 (0.60-0.66)	0.36 (0.35-0.41)	0.40 (0.38-0.43)	1.57 (1.50-1.74)	0.71 (0.67-0.79)	2.37 (2.28-2.67)
	Q2	0.60 (0.57-0.63)	0.41 (0.34-0.42)	0.39 (0.35-0.44)	1.60 (1.50-1.76)	0.73 (0.69-0.76)	2.49 (2.28-2.75)
	Q3	0.62 (0.58-0.66)	0.40 (0.35-0.42)	0.40 (0.36-0.45)	1.61 (1.54-1.77)	0.73 (0.65-0.76)	2.49 (2.38-2.73)
	Q4	0.61 (0.58-0.64)	0.40 (0.34-0.42)	0.41 (0.37-0.45)	1.63 (1.53-1.76)	0.69 (0.67-0.76)	2.50 (2.32-2.71)
	Q5	0.61 (0.57-0.65)	0.41 (0.35-0.42)	0.40 (0.38-0.46)	1.62 (1.52-1.81)	0.71 (0.68-0.73)	2.53 (2.41-2.78)
	p	0.163	0.317	0.521	0.647	0.554	0.350
FCH N = 96	Q1	0.58 (0.57-0.60)	0.40 (0.34-0.41)	0.36 (0.35-0.40)	1.56 (1.46-1.61)	0.73 (0.70-0.78)	2.45 (2.25-2.51)
	Q2	0.58 (0.56-0.61)	0.40 (0.34-0.41)	0.36 (0.35-0.40)	1.58 (1.47-1.66)	0.76 (0.70-0.79)	2.44 (2.25-2.53)
	Q3	0.51 (0.50-0.60)	0.51 (0.42-0.52)	0.36 (0.34-0.38)	1.85 (1.57-1.90)	0.76 (0.72-0.81)	2.89 (2.56-2.95)
	Q4	0.58 (0.55-0.59)	0.41 (0.35-0.42)	0.38 (0.34-0.39)	1.57 (1.46-1.66)	0.73 (0.70-0.78)	2.44 (2.24-2.62)
	Q5	0.58 (0.55-0.61)	0.41 (0.35-0.43)	0.41 (0.37-0.48)	1.63 (1.49-1.85)	0.71 (0.67-0.76)	2.54 (2.29-2.61)
	p	0.068	0.016	0.019	0.077	0.284	0.014
All subjects N = 288	Q1	0.61 (0.58-0.64)	0.36 (0.35-0.41)	0.39 (0.36-0.41)	1.56 (1.49-1.66)	0.73 (0.69-0.78)	2.39 (2.27-2.55)
	Q2	0.59 (0.57-0.62)	0.40 (0.34-0.42)	0.38 (0.35-0.41)	1.58 (1.49-1.69)	0.74 (0.70-0.77)	2.47 (2.27-2.64)
	Q3	0.60 (0.57-0.66)	0.41 (0.35-0.43)	0.39 (0.36-0.45)	1.61 (1.55-1.83)	0.74 (0.66-0.78)	2.51 (2.38-2.82)
	Q4	0.60 (0.57-0.63)	0.41 (0.35-0.42)	0.39 (0.36-0.42)	1.60 (1.51-1.70)	0.71 (0.67-0.77)	2.48 (2.32-2.64)
	Q5	0.60 (0.56-0.63)	0.41 (0.35-0.42)	0.40 (0.38-0.46)	1.62 (1.51-1.81)	0.71 (0.68-0.75)	2.54 (2.40-2.71)
	p	0.400	0.110	0.061	0.098	0.242	0.042

[a]Data (10^{-2}) are expressed as median (percentile 25-pertencile 75). Non-cholesterol sterols-to-total cholesterol levels are adjusted by gender, age, BMI and APOE genotype. *P* refers to statistical differences among quintiles.

[b]Synthesis markers are the sum of lanosterol and desmosterol levels.

[c]Phytosterols are the sum of campesterol, sitosterol, sitostanol and stigmasterol levels.

Table Supplementary 1. Clinical, biochemical and dietary characteristics of subjects among quintiles of "Vegetable & Fruits dietary pattern" scores.[a]

	Q1 N = 56	Q2 N = 57	Q3 N = 57	Q4 N = 57	Q5 N = 57	*p*
ADH/ FCH, %	17.4 / 24.5	20.0 / 20.2	21.6 / 17.0	22.1 / 16.0	18.9 / 22.3	0.433
Males, %	38 (67.9)	27 (47.4)	18 (31.6)	23 (40.4)	32 (56.1)	0.001
Age, years	37.7±9.48	44.8±9.85	45.1±7.83	48.0±9.82	48.4±8.87	< 0.001
Tobacco consumption, %						0.125
Smoker	22 (39.3)	19 (33.9)	13 (22.8)	16 (28.1)	7 (12.3)	
Former smoker	13 (23.2)	15 (26.8)	16 (28.1)	15 (26.3)	18 (31.6)	
Non smoker	21 (37.5)	22 (39.3)	28 (49.1)	26 (45.6)	32 (56.1)	
Packets/day x Years	16.5 (7.75-30.0)	23.0 (10.5-33.0)	20.0 (10.8-30.0)	13.0 (8.00-23.0)	20.0 (10.5-31.0)	0.489
Systolic blood pressure, mm Hg	127 (113-138)	130 (120-141)	126 (121-132)	134 (120-140)	130 (123-139)	0.219
Diastolic blood pressure, mm Hg	78.0 (70.0-87.0)	82.5 (73.0-88.0)	80.0 (77.0-87.0)	82.0 (76.0-88.0)	80.0 (79.5-84.0)	0.417
Body mass index, kg/m^2	25.8±3.41	25.1±2.11	24.9±1.88	25.2±2.74	25.3±2.54	0.768
Waist circumference, cm	90.7±12.7	88.5±8.18	87.0±8.65	88.9±10.5	90.0±11.1	0.676
Total cholesterol, mg/dL	306±55.4	296±42.1	307±46.5	296±33.8	315±55.6	0.534
HDL cholesterol, mg/dL	46.0 (39.0-58.0)	46.0 (36.8-54.0)	52.0 (44.0-64.5)	46.0 (38.0-63.0)	41.0 (37.0-56.5)	0.159
Non HDL cholesterol, mg/dL	243 (222-287)	245 (220-279)	241 (223-272)	234 (225-261)	270 (226-293)	0.741
LDL cholesterol, mg/dL	218 (192-250)	216 (189-247)	214 (190-242)	208 (196-227)	229 (184-269)	0.845
Triglycerides, mg/dL	145 (94.0-221)	130 (91.3-168)	133 (103-206)	179 (85.0-277)	270 (85.5-272)	0.892
Apolipoprotein A1,mg/dL	146±34.0	144±27.8	156±31.2	151±32.3	144±31.9	0.538
Apolipoprotein B, mg/dL	168±29.4	165±31.5	167±32.2	164±28.9	167±34.0	0.989
Lipoprotein(a), mg/dL	24.1 (12.0-55.0)	45.7 (15.5-69.2)	31.8 (13.0-75.2)	14.0 (5.27-37.1)	21.4 (9.54-48.7)	0.057

C reactive Protein, mg/L	1.55 (0.58-3.43)	2.75 (0.78-4.40)	1.30 (0.55-2.80)	2.60 (1.33-5.38)	2.00 (1.20-3.25)	0.108
GGT, IU/L	27.0 (18.0-38.0)	24.5 (17.8-32.0)	30.0 (15.5-50.5)	26.0 (18.0-65.0)	22.0 (15.5-38.0)	0.698
Glucose, mg/dL	88.7±10.2	90.2±10.3	87.5±9.73	93.6±11.0	92.8±15.0	0.181
HbA1c, %	5.10 (5.00-5.30)	5.40 (5.05-5.50)	5.20 (4.93-5.38)	5.30 (5.08-5.60)	5.30 (5.13-5.40)	0.053
Dietary intake						
Energy, kcal/day	2111 (1777-2466)	2104 (1695-2584)	2104 (1691-2545)	2093 (1803-2416)	2384 (2073-2879)	0.006
Carbohydrates, %	43.3±7.93	43.3±7.18	44.8±6.86	46.3±6.62	48.7±5.88	< 0.001
Protein, %	16.4±2.50	16.6±2.46	16.6±2.89	16.3±2.39	16.2±2.41	0.869
Fat, %	36.1±7.25	36.7±6.42	35.7±6.30	34.8±6.64	32.7±5.04	0.010
Monounsaturated fat, %	17.6±4.65	17.9±4.13	17.2±4.01	16.6±3.90	15.4±3.27	0.007
Polyunsaturated fat, %	4.30 (3.63-5.06)	4.69 (4.06-5.44)	4.66 (3.86-5.70)	4.53 (3.76-6.09)	5.01 (4.11-7.02)	0.056
Saturated fat, %	10.2±2.08	9.96±2.59	9.81±2.40	9.65±2.30	8.72±1.96	0.010
Cholesterol, mg/day[b]	347±129	328±109	349±101	314±89.7	358±125	0.219
Phytosterols, mg/day[b]	293 (230-340)	320 (257-391)	335 (274-410)	363 (314-417)	440 (374-520)	< 0.001
Fiber, g/day[b]	14.0 (12.3-16.9)	19.3 (16.6-22.1)	22.5 (20.0-26.5)	23.9 (21.8-29.1)	32.1 (28.2-37.0)	< 0.001
Alcohol, g/day[b]	6.74 (2.15-15.9)	8.23 (1.42-17.3)	5.08 (1.20-12.2)	4.38 (0.68-11.2)	4.38 (0.69-11.9)	0.296

[a]Data (10^{-2}) are expressed as mean±standard deviation or median (percentile 25-pertencile 75) as applicable. *P* refers to statistical differences among quintiles.

[b]Energy-adjusted.

Table Supplementary 2. Clinical, biochemical and dietary characteristics of subjects among quintiles of "Western dietary pattern" scores.[a]

	Q1 N = 56	Q2 N = 57	Q3 N = 57	Q4 N = 57	Q5 N = 57	*p*
ADH/ FCH, %	18.4/22.3	17.9/24.5	24.2/11.7	21.1/18.1	18.4/23.4	0.099
Males, %	19 (33.9)	31 (54.4)	25 (43.9)	29 (50.9)	34 (59.6)	0.061
Age, years	48.1±9.95	46.7±9.97	44.4±8.85	43.8±10.1	39.9±9.47	0.010
Tobacco consumption, %						0.021
Smoker	13 (23.2)	9 (15.8)	16 (28.6)	13 (22.8)	26 (45.6)	
Former smoker	19 (33.9)	17 (29.8)	18 (32.1)	15 (26.3)	8 (14.0)	
Non smoker	24 (42.9)	31 (54.4)	22 (39.3)	29 (50.9)	23 (40.4)	
Packets/day x Years	24.4 (8.50-36.0)	20.0 (8.75-34.3)	17.0 (10.0-32.8)	14.0 (9.13-22.9)	18.0 (10.0-31.0)	0.767
Systolic blood pressure, mm Hg	130 (120-134)	132 (122-140)	130 (120-139)	130 (123-139)	128 (117-138)	0.548
Diastolic blood pressure, mm Hg	80.0 (72.3-87.0)	83.5 (80.0-89.0)	80.0 (74.3-88.0)	82.0 (80.0-86.0)	80.0 (71.5-85.5)	0.219
Body mass index, kg/m^2	25.3±2.56	25.8±2.36	25.3±2.87	25.4±2.09	24.7±2.92	0.618
Waist circumference, cm	89.1±11.1	90.7±9.937	88.3±10.8	89.4±9.16	88.2±11.1	0.899
Total cholesterol, mg/dL	309±42.6	299±40.2	292±44.1	318±55.9	301±49.6	0.240
HDL cholesterol, mg/dL	47.0 (40.0-58.8)	45.0 (38.8-60.8)	49.0 (39.0-62.3)	52.0 (36.3-62.8)	43.0 (34.8-52.0)	0.529
Non HDL cholesterol, mg/dL	271 (221-284)	254 (222-271)	230 (213-251)	252 (227-297)	241 (223-283)	0.148
LDL cholesterol, mg/dL	226 (191-261)	226 (181-246)	202 (189-224)	218 (200-268)	222 (196-240)	0.566
Triglycerides, mg/dL	143 (94.0-277)	158 (98.0-210)	124 (89.8-185)	140 (89.8-235)	154 (94.0-300)	0.859
Apolipoprotein A1,mg/dL	153±28.7	156±31.6	151±28.1	147±34.8	136±32.0	0.097
Apolipoprotein B, mg/dL	174±31.7	167±28.9	159±33.3	168±30.2	164±28.6	0.365
Lipoprotein(a), mg/dL	20.5 (6.77-32.7)	26.8 (12.0-73.4)	25.7 (12.7-62.7)	16.0 (7.05-51.3)	43.5 (12.5-64.4)	0.208
C reactive Protein, mg/L	1.80 (0.60-4.10)	1.65 (0.50-2.50)	1.50 (0.70-3.55)	2.60 (0.95-4.03)	2.30 (0.90-3.85)	0.626
GGT, IU/L	25.5 (19.3-50.3)	30.5 (16.0-48.8)	25.5 (16.0-33.8)	21.5 (15.5-51.3)	26.0 (18.0-32.0)	0.818

Glucose, mg/dL	89.3±9.00	91.8±10.3	90.4±13.0	89.6±13.9	91.3±10.1	0.907
HbA1c, %	5.30 (5.10-5.48)	5.25 (4.88-5.50)	5.20 (5.00-5.40)	5.30 (5.10-5.40)	5.20 (5.10-5.48)	0.765
Dietary intake						
Energy, kcal/day	2054 (1684-2341)	2091 (1680-2600)	1940 (1659-2244)	2364 (1972-2701)	2454 (2140-2865)	< 0.001
Carbohydrates, %	42.9±7.87	45.3±6.89	44.1±6.81	47.4±6.38	46.7±7.09	0.004
Protein, %	16.4±2.62	16.7±2.65	17.4±2.44	16.2±2.28	15.5±2.34	0.002
Fat, %	37.1±7.41	34.8±6.12	35.4±6.39	33.9±5.37	34.8±6.71	0.100
Monounsaturated fat, %	18.6±5.02	17.1±3.69	17.2±3.93	15.8±3.48	16.1±3.67	0.002
Polyunsaturated fat, %	4.98 (4.05-5.52)	4.57 (3.89-5.38)	4.43 (3.74-5.85)	5.57 (3.71-5.68)	4.72 (3.80-7.32)	0.706
Saturated fat, %	9.29±2.31	9.42±2.18	9.76±2.45	9.78±2.22	10.0±2.42	0.451
Cholesterol, mg/day[b]	302±101	338±107	311±95.1	360±118	384±119	< 0.001
Phytosterols, mg/day[b]	341 (284-405)	354 (266-430)	311 (269-359)	377 (320-447)	389 (316-470)	0.002
Fiber, g/day[b]	22.5 (17.7-26.6)	21.9 (18.7-29.6)	22.1 (16.7-27.7)	23.5 (19.3-31.9)	21.6 (15.8-28.0)	0.344
Alcohol, g/day[b]	4.42 (0.68-15.5)	5.08 (0.35-16.3)	5.14 (1.42-15.2)	4.38 (1.46-11.8)	5.92 (1.79-15.6)	0.811

[a]Data (10^{-2}) are expressed as mean±standard deviation or median (percentile 25-pertencile 75) as applicable. *P* refers to statistical differences among quintiles.

[b]Energy-adjusted.