INVESTIGATION OF PLANT STEROLS, OXIDIZED LOW DENSITY LIPOPROTEINS (LDL) AND HOMOCYSTEINE LEVELS IN PATIENTS WITH CORONARY ARTHERY DISEASE AND HEALTHY CONTROLS

Abdullah Sivrikaya PhD,[1] İdris Mehmetoğlu Prof. PhD,[2] Sevil Kurban Assist. Prof. MD,[2] Nazif Aygül Assist. Prof. MD,[3] F. Hümeyra Yerlikaya PhD[2]

[1] Selcuk University, Selcuklu Faculty of Medicine, Department of Biochemistry, Konya, Turkey
[2] Selcuk University, Meram Faculty of Medicine, Department of Biochemistry, Konya, Turkey
[3] Selcuk University, Selcuklu Faculty of Medicine, Department of Cardiology, Konya, Turkey

ABSTRACT

• ***Objective:*** *This study was performed to demonstrate the role of serum plant sterols (β-sitosterol, stigmasterol) in the development of coronary artery disease (CAD) and the correlation of them with other potential risk factors.*

• ***Material and Method:*** *Totally 166 subjects who underwent angiography were classified according to their coronary angiography results. Control group consisted of 60 subjects with normal coronary angiograms. Patient group consisted of 106 subjects with ≥ 50% stenosis in at least one of three major coronary arteries. Serum β-sitosterol, stigmasterol, oxidized low density lipoprotein (Ox-LDL) and homocysteine levels were measured.*

• ***Results:*** *Serum β-sitosterol, stigmasterol, their ratios to cholesterol, Ox-LDL and homocysteine levels of patients were slightly but not significantly higher than those of controls. Serum plant sterol levels of three patients were very high compared to those of the other subjects. There was a significant positive correlation between β-sitosterol and cholesterol in the patients group and between stigmasterol and cholesterol levels in the control group. However, there was no correlation between serum plant sterols levels and those of Ox-LDL, homocysteine and the degree of coronary occlusion.*

• ***Conclusion:*** *Our results show that serum plant sterols are not independent risk factors for CAD. However, routine measurement of serum plant sterols in addition to other risk factors seems to be very important for a more reliable evaluation of patients with CAD.*

• ***Key Words:*** *β-sitosterol, stigmasterol, coronary artery disease, oxidized low density lipoprotein, homocysteine*
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SAĞLIKLI KİŞİLERDE VE KORONER ARTER HASTALARINDA BİTKİ STEROLLERİ, OKSİDE LDL VE HOMOSİSTEİN DÜZEYLERİNİN ARAŞTIRILMASI

ÖZET

• **Amaç:** Bu çalışma serum bitki sterollerinin (β-sitosterol, stigmasterol) koroner arter hastalığının gelişimindeki rolünü ve diğer potansiyel risk faktörleriyle korelasyonunu göstermek için yapıldı.

• **Materyal ve Metod:** Anjiografisi yapılan toplam 166 kişi koroner anjiografi sonuçlarına göre kontrol ve hasta olarak gruplandırıldı. Anjiografisi normal olan 60 kişi kontrol grubunu, üç majör koroner arterlerinin en az birinde ≥ %50 stenozu olan 106 kişi hasta grubunu oluşturdu. Vakaların serum β-sitosterol, stigmasterol, okside LDL ve homosistein düzeyleri analiz edildi.

• **Bulgular:** Hastaların serum β-sitosterol, stigmasterol ve bunların kolesterole oranları, okside LDL ve homosistein düzeyleri kontrollere göre biraz yüksek (istatistiksel açıdan önemsiz) bulundu. Üç hastanın serum bitki sterol seviyeleri diğerlerine göre çok yüksekti. Hasta grubunda β-sitosterol ile kolesterol düzeyleri arasında, kontrol grubunda stigmasterol ile kolesterol düzeyleri arasında önemli pozitif korelasyon vardı. Serum bitki sterolleri ile bunların okside LDL, homosistein ve koroner oklüzyon seviyeleri arasında ise korelasyon yoktu.

• **Sonuç:** Bulgularımız serum bitki sterollerinin koroner arter hastalarında bağımsız risk faktörü olmadığını göstermektedir. Ayrıca bu hastalarda diğer risk faktörlerinin yanında serum bitki sterollerinin de rutin olarak ölçülmesinin hastaların daha iyi değerlendirilmesinde çok önemli olacağı kanaatindeyiz.

• **Anahtar Kelimeler:** β-sitosterol, stigmasterol, koroner arter hastalığı, okside LDL, homosistein. **Nobel Med 2010; 6(3): 39-45**

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease of large and medium-sized arteries with hardening and loss of elasticity of the arterial walls and narrowing of the arterial lumen, and involving accumulation of lipids, lipoproteins, and mononuclear cells (monocytes and T-cells) in the subendothelial space of blood vessels.[1,2] Coronary artery disease (CAD) which leads to mortality develops at the end of atherosclerosis.[3]

During the past 50 years, much evidence has documented a relationship between diet and CAD risk.[4] Very high amount of plant sterols (several grams per day) are believed to reduce plasma cholesterol concentrations by competitively blocking cholesterol absorption and thus protect against atherosclerosis.[5] However, sitosterolemia is a rare autosomal recessively inherited plant sterols (phytosterols) storage disease in which significantly increase of plant sterols are found in blood and various tissues of the patients.[6-8] Sitosterolemia is characterized by a strong propensity toward premature coronary atherosclerosis. On the contrary, serum cholesterol level may be normal in these patients.[6,8,9]

Plant sterols are not synthesized endogenously in humans, including patients with sitosterolemia, but are derived entirely from the diet. Since plant sterols are not metabolized, their plasma concentrations depend on their intestinal absorption and biliary elimination rates. The rates of absorption vary among the individual plant sterols.[9-13]

The liver preferentially excretes plant sterols over cholesterol. Dietary sterols have recently been shown to passively enter intestinal cells, and subsequently the vast majority are pumped back into the gut lumen by ATP-binding cassette (ABC) transporter proteinssterols.[12-14] Sitosterolemia has recently been shown to result from mutations in either of the genes for two proteins (ABCG5 or ABCG8). The active pumping back into the intestine of passively absorbed plant sterols is disrupted, and hepatic secretion of the resultant accumulation of these sterols is decreased. The ability of the liver to preferentially excrete plant sterols into the bile is apparently impairedsterols.[7,8,12-15]

The normal daily phytosterol intake ranges from 167 to 437 mg in various populations. However, when consumed daily in much larger quantities, plant sterols and stanols inhibit cholesterol absorption also by disrupting the micellar solubilization of cholesterol.[16] Both oral and parenteral administration of plant sterols results in reduced concentrations of plasma cholesterol. This reduction may be due not only to the inhibition of intestinal cholesterol absorption but also to other effects on hepatic and intestinal cholesterol metabolism.[17]

Oxidative modification of lipoproteins may play a key role in the pathogenesis of atherosclerosis. These modifications accelerate atherosclerotic lesion development, induce expression of proinflammatory cytokines, impair vasodilatation and produce toxicity to endothelial cells.[18,19] Oxidative stress occurs when the equilibrium between antioxidant defense system→

and free radicals degenerates.[20,21] Elevated oxidative stress and superoxide anion formation in vascular cells could promote conversion of low density lipoprotein (LDL) to atherogenic oxidized LDL (Ox-LDL) which plays an important role in the pathogenesis of atherogenesis.[18,19,22]

Another significant risk factor for CAD is homocysteine. It has been estimated that 10% of the population risk for cardiovascular disease in the United States is associated with increased total homocysteine. Even, increased plasma total homocysteine is linked to increased risk of cardiovascular disease to a similar extent to that of smoking and hyperlipidemia.[23] The elevation of homocysteinemia causes an increased formation of H_2O_2 and a decreased activity of glutathione peroxidase, superoxide dismutase and catalase, the principal antioxidant enzymes, thus promoting the generation of oxidative stres.[24]

Although hypercholesterolemia, Ox-LDL and homocysteine levels are well defined as major risk factors in CAD, the role of serum plant sterols in the development of CAD is somewhat contraversial and the association of them with the above risk factors is not known.

Therefore, in this study, our aim was to demonstrate the role of serum plant sterols in the development of CAD documented by angiography and the correlation of them with the degree of coronary artery occlusion and other potential coronary risk factors.

MATERIAL and METHOD

The study was performed on 166 subjects (120M, 46F) aged 28-87 (60.9±11.8) years after approval of the ethics committee. The subjects were selected among those applied to the emergency department with complaints of chest pain. After the first evaluation, those decided to underwent angiography in the department of cardiology were included in the study. Then subjects were classified according to their coronary angiography findings as follows: Control group consisted of 60 subjects (38M, 22F, aged 59.8±12.9 years) with normal coronary angiograms (no vessel disease) and with less than 50% stenosis in any of the three major coronary arteries.

Patients group consisted of 106 subjects (82M, 24F, aged 62.8±11.0 years) and with ≥ 50% stenosis in at least one of the three major coronary arteries. Details of the subjects were recorded at the time of admission including age, sex, height, weight, alcohol consumption, cigarette smoking, diabetes mellitus, hypertension and family history. None of the patients was vegeterian. Baseline characteristics of the study subjects are shown in Table 1.

Table 1: Baseline characteristics of the study subjects

Characteristics	Patients (n=106)	Controls (n=60)	P value
Male/Female (n, % / n, %)	82 (77.34)/24 (22.64)	38 (63.33)/22 (36.67)	NS
Age (year)	62.8±11.0	59.8±12.9	NS
Smoking (%)	49 (46.23)	13 (21.67)	0.002
Family history (%)	15 (14.15)	7 (11.67)	NS
Diabetes (%)	24 (22.64)	6 (10.00)	0.042
Hypertension (%)	52 (49.06)	22 (36.67)	NS
Hypertension+Diabetes (%)	16 (15.09)	4 (6.67)	NS
Stable AP (%)	18 (16.98)	19 (31.67)	0.029
Unstable AP (%)	39 (36.79)	37 (61.67)	0.002
STEMI (%)	49 (46.23)	4 (6.67)	<0.001

AP: Angina pectoris; STEMI: ST Elevation Myocardial Infarction; NS: Not significant. The values of age are presented as mean ± standart deviation ($\bar{x}$± SD).

Totally 8-10 ml of blood samples were drawn after a 12-14 hours of fasting in the morning. Sera of the samples were separated after coagulation and stored at -85°C until the day of analysis. Serum plant sterols (stigmasterol, β-sitosterol), Ox-LDL, homocysteine and lipids (total-cholesterol, triglyceride, high density lipoprotein (HDL) cholesterol and LDL-cholesterol) levels were measured.

Determination of serum plant sterols

Serum β-sitosterol and stigmasterol concentrations were assayed by high-performance liquid chromatography (HPLC) technique after derivatization with benzoyl chloride as described by Kasama et al.[25] with slight modifications. Briefly, 0.1 ml of serum was treated with 1 M ethanolic KOH in a screw-capped test-tube. After vigorous stirring, the mixture was allowed to stand for 1 h at 80°C, after which 0.5 ml of water was added to the saponified mixture. The unsaponifiable material was extracted with two 2 ml portions of hexane. The extracts were pooled in another screw-capped test-tube and evaporated under a stream of nitrogen. The benzoyl chloride reagent which was freshly prepared for each assay (0.3 ml), was added to the test-tube containing the unsaponifiable material. After vigorous stirring, the solution was allowed to stand for 30 min at room temperature. After addition of 2 ml of 1,2- dichloroethane, the reaction mixture was washed successively with 2 ml of 0.1 M hydrochloric acid and twice with 2 ml of water. The organic layer was evaporated under a stream of nitrogen. The residue was dissolved in 0.5 ml of acetonitrile-1,2-dichloroethane (2:1, v/v) and 20 µl was injected into the HPLC system. The solvent used for the elution was acetonitrile-water-acetic acid (97:3:0.2,→

Table 2: Serum lipid profile, stigmasterol, β-sitosterol, stigmasterol/cholesterol ratio, β-sitosterol/cholesterol ratio, Ox-LDL and homocysteine levels of the groups

Parameters	Patients (n=106)	Controls (n=60)	P
Total-cholesterol (mg/dl)	172.4 ± 4.2	167.4 ± 5.7	NS
Triglyceride (mg/dl)	135.3 ± 8.3	124.5 ± 12.5	NS
HDL-cholesterol (mg/dl)	36.5 ± 1.0	39.0 ± 1.3	NS
LDL-cholesterol (mg/dl)	109.1 ± 3.4	102.6 ± 4.5	NS
Stigmasterol (µg/ml)	5.02 ± 1.21	2.86 ± 0.48	NS
β-sitosterol (µg/ml)	5.59 ± 1.39	3.22 ± 0.37	NS
Stigmasterol/total-cholesterol ratio (µg/mg)	4.79 ± 1.12	2.90 ± 0.52	NS
β-sitosterol/total-cholesterol ratio (µg/mg)	5.25 ± 0.96	3.46 ± 0.43	NS
Ox-LDL (U/L)	119.7 ± 4.7	114.5 ± 4.1	NS
Homocysteine (µmol/L)	17.62 ± 0.78	16.61 ± 0.60	NS

HDL-cholesterol: High density lipoprotein cholesterol; LDL-cholesterol: Low density lipoprotein cholesterol; Ox-LDL: Oxidized LDL; NS: not significant. The values of parameters are expressed as mean ± standard error ($\bar{x}$±SE).

Table 3: The correlations between the parameters of the patients group

Parameters	Cholesterol	Stigmasterol	β-sitosterol	Ox-LDL	Homocysteine
Gensini Score	0.064	-0.109	-0.060	0.032	-0.033
Homocysteine	0.017	0.008	-0.033	-0.096	
Ox-LDL	0.170	0.077	0.103		
β-sitosterol	0.176*	0.717**			
Stigmasterol	0.156				

* p< 0.05 ** p< 0.01

Table 4: The correlations between the parameters of the control group

Parameters	Cholesterol	Stigmasterol	β-sitosterol	Ox-LDL
Homocysteine	-0.058	-0.038	0.038	-0.048
Ox-LDL	0.194	0.010	-0.067	
β-sitosterol	0.174	0.705**		
Stigmasterol	0.317*			

* p< 0.05 ** p< 0.01

v/v) at a flow-rate of 2 ml/min. The separation of the sterols was performed on a reverse-phase column (Sigma: 4.6x250 mm, ODS-2, C18, 5µm) maintained in an incubator at 50°C and monitored at 228 nm. A standart cromatograms of stigmasterol and β-sitosterol are shown in Figure.

Plant sterols are transported in serum by lipoproteins, changes in lipoprotein concentrations also affect concentrations of serum plant sterols. Therefore, we have expressed serum plant sterols in concentrations as well as in ratios to cholesterol.[26] For this purpose, cholesterol levels were also analyzed by HPLC in the same run as the serum plant sterols.

Determination of serum homocysteine: Serum homocysteine levels were measured by HPLC (agilent 1100) using a commercial available kit (Chromsystems, Cat no:503).

Determination of serum Ox-LDL: Serum Ox-LDL levels were determined by using a commercially available kit (Mercodia Oxidized-LDL ELISA kit, Cat no: 13537).

Determination of serum lipids: Total-cholesterol, triglyceride and HDL-cholesterol levels were measured by routine methods on Beckman Coulter LX20 autoanalyzer. LDL-cholesterol levels were calculated by Friedewald Formula.[27]

Statistical analysis

Statistical analysis were done with the Statistical Package for the Social Sciences (SPSS, Version 10.0). Student's t test or χ^2 analysis was used to assess various baseline characteristics. The distribution of the variables studied using a Lilliefors test for normality, and then we used the Student's t-test for normally distributed data (Ox-LDL and lipid findings) and Mann-Whitney U-test for non-normally distributed data (stigmasterol, β-sitosterol and homocysteine). The correlations between variables were performed by Pearson's Correlation test. Data were expressed as mean ± standard error ($\bar{X}$± SE). Results were considered statistically significant at $p<0.05$.

RESULTS

The findings are given in Table 2, and the correlations between the parameters are given in Table 3 and 4. Serum β-sitosterol, stigmasterol, the ratios of them to cholesterol (measured by HPLC), homocysteine and Ox-LDL levels of the patients were slightly but not significantly higher than those of the controls. There was a significant positive correlation between β-sitosterol and cholesterol ($r= 0.176$, $p<0.05$) in the patients group and between stigmasterol and cholesterol ($r= 0.317$, $p<0.05$) in the control group.

Also, there was no significant correlation between serum plant sterols levels and the levels of Ox-LDL, homocysteine and the degree of coronary occlusion (Gensini score).

On the other hand, one of the most important finding of this study was the determination of very high level→

of serum plant sterols of three patients in spite of their normal total-cholesterol levels compared to those of the other patients and the controls.

The findings of those patients were as follows

Patient 1: Serum total-cholesterol, stigmasterol and β-sitosterol levels were 240mg/dl, 83.3µg/ml and 140.5µg/ml respectively. In that patient, there was a 50% stenosis in one major epicardial coronary artery, unstable angina pectoris (AP), positive family history and hypertension.

Patient 2: Serum total-cholesterol, stigmasterol and β-sitosterol levels were 147mg/dl, 93.0µg/ml and 21.4µg/ml respectively. In that patient there was a >50% stenosis in one major epicardial coronary artery, stable AP and hypertension.

Patient 3: Serum total-cholesterol, stigmasterol and β-sitosterol levels were 179mg/dl, 9.36µg/ml and 46.4µg/ml respectively. In that patient, there was a >50% stenosis in two major epicardial coronary artery, unstable AP, myocardial infarction during the past, hypertension and cigarette smoking.

As seen, serum plant sterol levels of those 3 patients were very high compared to those of the patients and the controls. Also, the levels are very similar to those previously found in patients with sitosterolemia.[28,29]

DISCUSSION

Our study is the first one investigating serum plant sterol levels and correlation between these parameters and serum Ox-LDL and homocysteine levels in patients underwent angiography.

The measurement of serum plant sterols in patients with CAD living in different regions may provide valuable information about the incedence of the disease. Because, sitosterolemia is a rare genetic disorder. Therefore, there might be significant difference between the occurance of the disease in different ethnic populations. Also, environmental factors and dietary habits may play an important role in the absorption of the plant sterols.

In our study, serum plant sterol levels of the patients with CAD group was slightly, but not significantly higher than those of the control group.

The findings concerning the role of serum plant sterol levels in CAD are contraversial. Indeed, Assmann et al.[9], Sudhop et al.,[12] Guleck et al.,[29] Rajaratnam et al.[30] and Matthan et al.[31] have reported an important significant association between serum plant sterol levels and CAD.

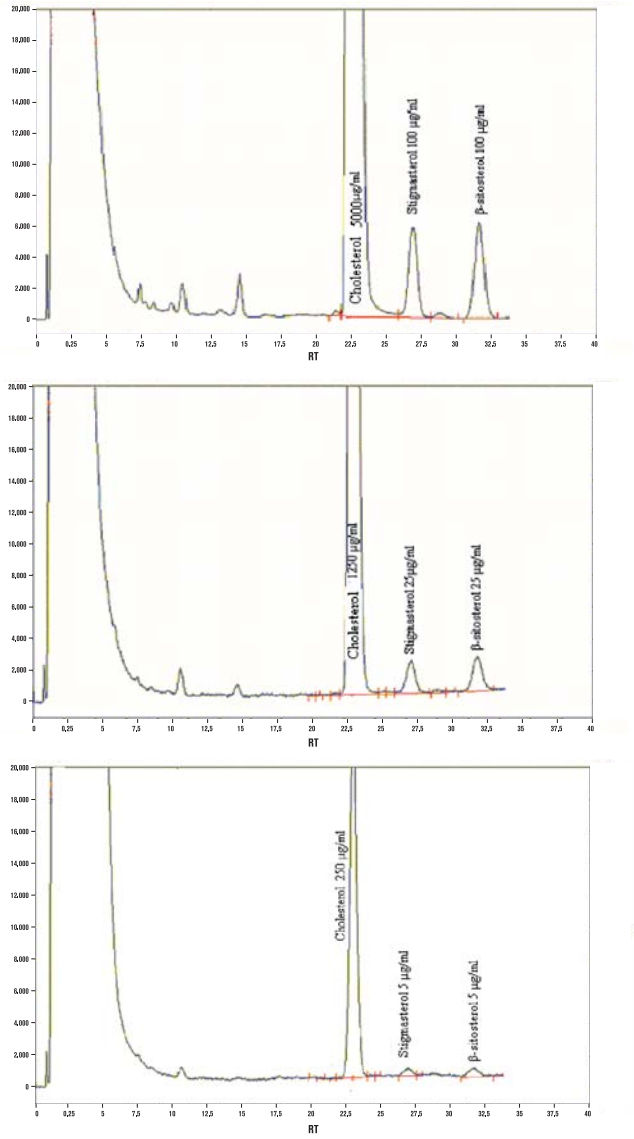

Figure. The standart cromatograms of stigmasterol and β-sitosterol in our study determined by HPLC

On the other hand, Kuriyama et al.,[6] Pinedo et al.[26] and Wilund et al.[32] have found no correlation between serum plant sterol levels and CAD. The findings of these investigators are in accordance with our findings. The difference between these findings suggests that such factors as genetic, environmental and nutritional habits may results in different findings of various investigators. Also some other unknown factors may lead to the difference between the findings of various investigators.

On the other hand, we have found that serum plant sterol levels of three patients were very significantly higher (about 10 to 20 times) while total-cholesterol levels were not significantly different compaired to those of the other patients and the control group and those reported in the literature.[6,8,9] The underlying mechanism of that finding was not known. However, it may be regarded as a sign of sitosterolemia in those patients because their serum plant sterol levels were similar to→

those of patients with sitosterolemia found in the literature.[8,10] However, we could not evaluate them further because they were discharged from the hospital when we completed our study and obtained serum plant sterols results. Very high levels of serum plant sterols in three patients among 166 subjects seems to be surprising regarding to the incedence of the pathologic levels of serum plant sterols in such a few number of subjects. However, our hospital is a tertiary care reference hospital in the surrounding area. Therefore, the three patients might previously have applied to the other hospitals in the area and have been diagnosed in our hospital finally. Thus they can be regarded as representing a larger number of population that those we have included in our study.

Also, this is the first study in our region and even in our country measuring serum plant sterol levels in patients with CAD. Therefore, we don't know if there is a genetic tendency toward increased absorption of plant sterols in our subjects.

We have found a significant positive correlation between serum β-sitosterol and cholesterol levels in the patients group and a positive correlation between stigmasterol and cholesterol levels in the control group. This is an unexpected finding because plant sterols are known to reduce cholesterol absorption at high levels.[5,16,26,33,34] Although, this finding suggests that plant sterols may contribute to the development of CAD, the absence of difference between serum plant sterol levels of our subjects do not support this idea. Therefore, we could not explain the underlying mechanism of this finding.

In our study, serum Ox-LDL and homocysteine levels of the patients group were slightly, but not significantly, higher than those of the control group. Also, there was no significant correlation between the levels of these parameters and those of stigmasterol and β-sitosterol of the subjects. This shows that serum plant sterols especially stigmasterol and β-sitosterol have no impact on the formation of Ox-LDL and the metabolism of homocysteine.

In the literature generally Ox-LDL and homocysteine levels of patients with CAD are found to be higher than those of the healty controls.[35-39] Therefore, our findings concerning these two parameters are not consistant with the findings of the literature. However, our control group has beeen selected among patients with some complaints similar to those of patients with CAD and therefore they have underwent to angiogarphy. Thus, our control group may also have somewhat high levels of Ox-LDL and homocysteine compared to those of subjects with no complaints. This may provide an explanation to our finding.

CONCLUSION

In conclusion, our results showed that serum plant sterols are not independent risk factors for CAD. Also, there was no correlation between serum plant sterols and Ox-LDL and homocysteine levels in our subjects. However, routine measurement of serum plant sterols in patients with CAD may be advised to determine patients with possible sitosterolemia and to treat them accordingly.

C **CORRESPONDING AUTHOR: Abdullah Sivrikaya PhD.** Selçuk Üni, Selçuklu Tıp Fakultesi, Biyokimya AD, Alaeddin Keykubad Kampusü, Konya **biyokaya@selcuk.edu.tr**

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