# Akut ve Kronik Sigara İçen Genç Bireylerde Proinflamatuar Durumda HDL Fonksiyonlarının Önemi Nedir?

## *What's the Importance of HDL Functions in Proinflammatory Status of Young Subjects in Both Acute and Chronic Smoking?*

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**ÖZET**

**Amaç:** Sigara içimi, kardiyovasküler hastalıkların en önemli ve önlenebilir risk faktörlerinden biridir. Okside LDL, kardiyovasküler hastalıklarda artış gösterir. Normal yüksek dansiteli lipoprotein (HDL), LDL'yi oksidasyondan koruyabilirken proinflamatuar HDL bunu yapamamaktadır. Bu çalısmanın amacı, hem akut hem de kronik olarak sigara içen genç bireylerin, sigara içmeyenlere göre kardiyovasküler hastalıklara yatkın olan proinflamatuar HDL'ye sahip olup olmadıklarını belirlemekti.

**Gereç ve Yöntem:** Bu çalısmaya yaklasık 8-10 yıldır sigara kullanan 40 saglıklı birey ve sigara kullanmayan 40saglıklı birey dahil edildi. Kan örnekleri bir gecelik açlık ve sigara yoklugu durumunda ve sigara içiminden 1 saat sonra toplandı. LDL'nin oksidasyondan korunmasında HDL'nin antioksidan yetenegi ölçüldü. Aynı zamanda total kolesterol, trigliserid, HDL kolesterol, LDL kolesterol, Apo A-1, Apo B, hsCRP ve Lp(a) rutin standart metodlar kullanılarak klinik laboratuarımızda belirlendi.

**Bulgular:** Total-C, LDL-C, Lp(a) ve CRP düzeyleri sigara içen ve kontrol grupları arasında farklı değildi. Kontrol grubuna göre sigara içen grupta plazma HDL-C seviyesi düşük bulunurken, TG ve Apo B seviyeleri anlamlı derecede yüksekti. Sigara içenler yüksek oranda proinflamatuar HDL'ye sahiplerdi. Sigara içenlerin proinflamatuar HDL oranı %80, sigra içmeyenlerin %10 iken; sigara içimi öncesi bu oran %48, sigara içimi sonrası ise %68 idi.

**Sonuç:** Sigara içimi, HDL'nin antiinflamatuar fonksiyonlarını zayıflatmıstır. Bu sonuç, sigara içiminin proinflamatuar HDL düzeylerini artırarak ateroskleroz prosesinde rolü oldugunu göstermektedir.

**Anahtar Kelimeler:** Ateroskleroz; oksitlenmiş düşük dansiteli lipoprotein; yüksek yoğunluklu lipoprotein-1; DCF-H

**ABSTRACT**

**Objective:** Smoking is one of the most important cause of cardiovascular disease and known as a preventable risk factor. Lipoproteins especially LDL has an important role in cardiovascular disease. Normally high density lipoproteins protect LDL from oxidation; proinflammatory HDLs do not. The aim of this study was to determine whether the young smokers who smoke acute or chronically, have more proinflammatory HDL which may predispose them to cardiovascular disease compared to nonsmokers.

**Materials and Methods:** Forty young smokers, who have been smoking for 8-10 years and forty healthy nonsmokers were included in this study. Blood samples were collected after overnight fast with on absence of smoking and one hour after smoking a cigarette. The ability of the subjects HDLs to prevent oxidation of LDL-C was measured. Total-C, TG, HDL-C, LDL-C, Apo A, Apo B, hsCRP and Lp(a) levels were also determined using routine standard methods.

**Results:** Total-C, LDL-C, Lp(a) and hsCRP levels were not different between the smokers and control groups. The plasma level of HDL-C was significantly lower and those of TG, and Apo B levels were significantly higher in the smoking group than in the control group. The smokers had higher proinflammatory HDL compared to those of nonsmokers (80% vs 10%). Antiinflammatory HDL levels of smokers were decreased after smoking (48% and 68% respectively).

**Conclusion:** Chronic smoking impairs HDLs anti inflammatory functions and balance between lipoproteins and apolipoproteins. This result may indicate that proinflammatory HDL induced by smoking may have a role in atherosclerosis process.

**Keywords:** Atherosclerosis; oxidized low density lipoprotein; high density lipoprotein-1; DCF-H

## INTRODUCTION

Cigarette smoking is generally accepted as the most important cause of death in the Turkey as well as all over the world (1). Also atherosclerosis which results from different risk factors including smoking, is the most important cause for coronary artery disease (CAD) and accepted as an inflammatory disease. The lipogenic hypothesis states that both lesion initiation and progression in atherosclerosis appear to be associated with increased levels of plasma oxidized LDL (2). Oxidized LDL; stimulates the expression of VCAM-1 (vascular cell adhesion molecule-1) on endothelial cells, which is directly chemotactic for monocytes, increases the expression of MCP-1 (monocyte chemotactic protein-1) by vascular cells, and stimulates macrophage proliferation (3). These properties could promote the early inflammatory infiltration of monocytes into the vascular wall which contributes the process of atherosclerosis (4).

The mechanism of smoking-induced endothelial dysfunction is unclear and complex mechanism. Smoking manifests its direct toxic effect on human endothelial cells by reducing endothelial prostacyclin production and increasing leukocyte adhesion to endothelial cells (5).

Numerous epidemiological studies have associated HDL with an inverse risk for coronary artery disease (6). High density lipoprotein cholesterol levels in human plasma correlate generally with protection against coronary heart disease, an effect believed to be due to multiple protective actions of HDL including stimulating the removal of excess cholesterol from cells and reducing inflammation in the artery wall (7). The initial formation of HDL particles requires the membrane lipid transporter ATP-binding cassette transporter A1 (ABCA1) to mediate delivery of cellular lipids to HDL apolipoproteins.

This reduces excess cholesterol stores in cells including arterial wall macrophages (7). This 'protective' effect of HDL may be due in part to inhibition of the oxidative modification of LDL or by promoting cholesterol efflux from peripheral cell (8). In recent studies, it was shown that HDL's antioxidant functions became as important as other known functions like esterification of free cholesterol, activation of lecithin cholesterol acyltranspherase (LCAT) and lipoprotein lipase enzymes, reverse cholesterol transport and removing

lipoprotein remnants from circulation (9-10). Paraoxanase (PON) which is in HDL and binding to $Ca^{+2}$ has important roles on inhibiting LDL oxidation and decreasing activity of toxic metabolites occuring in LDL oxidation. In vitro studies showed that PON, hydrolized oxide-LDL and decreased lipid hydroperoxide and prevented fatty streaks (11). Also PON is bound with Apo A1 in hydrophobic region in amino end and decreases risk of vascular disease by rupturing proinflammatuar molecules which results from LDL oxidation (12). On the other hand, ıt was shown that significant amount lysophosphatidyl choline was found in HDL oxidation and PON-1 degradated phosphatidylcholine as an phosphalipase A2 enzyme (13). Therefore, in the recent studies it was emphasized that HDL's antioxidant capacity is more important than it's concentration in preventing atherosclerosis (14).

In this regard we aimed to study the pro-inflammatory/anti-inflammatory properties of HDL in young smokers and non-smokers for assesment of cardiac risk and atherosclerosis progress among smokers. The second aim of this study was to determine the acute effect of smoking on proinflamatuar/ antiinflammatuar properties of HDL in young smokers.

## MATERIALS AND METHODS

This study was carried out in accordance with the Helsinki Declaration and approved by the ESOGU Medical faculty local ethics committee. Volunteers eligible for this study were men and women aged from 23 to 33 which were healthy and young. Control group was consist of healthy nonsmokers (n=40). Nonsmokers were classified as either never smokers or ex-smokers (more than 5 years). Forty smokers (9 pack year) have a smoking duration 8-10 years. The number of pack-years of smoking was derived by using smoking intensity and duration, i.e. number of packs of cigarettes per day multiplied by the number of years of smoking (1 pack=20 cigarettes). Smokers gruop was consist of 16 female and 24 males (Table 1). After 10-12 hours fasting with on absence of smoking and one hour after smoking a cigarette for acute smoking blood samples were collected and serum levels of total cholesterol (TC), triglycerids (TG), HDL, LDL, Apo A, Apo B and hsCRP were measured.

TC, HDL-C, LDL-C, TG, concentrations were measured by enzymatic colorimetric method but Lp(a) and hsCRP concentrations were measured by immunoturbidimetric method by Roche Diagnostics kits according to manufacturer suggestions in Modular Roche analyzer.

To determine the functional properties of HDL, the change in fluorescence intensity resulting from oxidation of DCFH by LDL in the presence of test HDL was measured (12).

### LDL Isolation

LDL, isolated from routine serum samples of our clinical laboratory were used for the determination of abilitiy of HDL to protect against LDL oxidation (15-16).

**Table 1.** Characteristics of young subject

| | **Control (n=40)** | **Smokers (n=40)** | **p value** |
|---|---|---|---|
| Age | 26.62±2.94 | 27.17±3.07 | p=0,416 |
| Gender | | | p=0,344 |
| Male | 40.7 % | 59.3 % | |
| Female | 54.7 % | 45.3 % | |

*$\chi^2$ (YATES)

Serums and precipitation reagents were allowed to equilibrate to room temperature. 500 μl of the serum was added to 5 ml of the heparin-citrate buffer. After mixing with a vortex mixer, 5 ml of the suspension was removed for cholesterol determination and the remainder was allowed to stand for 10 min at room temperature. The insoluble lipoproteins were then sedimented by centrifugation in a centrifuge at 3400 rpm for 10 min at 25 C. The final pH was 5.11. LDL-C concentration was determined with Modular Roche.

**HDL Isolation**

Isolation of HDL from volunteers samples were carried out by Dextran Sulphate precipitation method (17).

**Dichlorofluorescein (DCF) Assay**

DCFH-DA was dissolved in fresh methanol at 2,0 mg/ml and was incubated at room temperature and protected from light for 30 min. This results in the release of DCFH. On interaction with lipid oxidation products DCFH forms DCF, which produces intense fluorescence (18). 200 μl of the isolated LDL solution (final concentration 50 μg/ml) and 900 μl of test HDL (at a final concentration of 10 μg/ml cholesterol) were incubated in a tube at 20-25 °C and in a dark room for 1 hour. 100 μl of DCFH solution (0.2 mg/ml) was then added to each well and incubated for 2 hours more. Fluorescence intensity was determined with spectrofluorometer (Jasco FP-750) set at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Values of fluoresence units (FU) (HDL Score) were normalized to 1.0 as the positive control. Values >1.0 FU after the addition of test HDL indicated as proinflammatory HDL; values <1.0 indicated as antiinflammatory HDL (18).

**Statistical analyses**

We used Statistical Package for the Social Science (SPSS) for Windows 13.0 for statistical analysis. Continuous variables summarized with n (sample size), mean and standard deviation, categorical variables summarized with n (sample size), median and $25^{th}$ $75^{th}$ percentiles. Normally distributed continuous variables were compared between groups with student t test. Normally distributed continuous dependent variables were analyzed paired t test. Nonnormally distributed variables were compared with Mann Whitney U test for independent groups, Wilcoxon test for dependent two groups. $Chi\text{-}square_{(Yates)}$ analyses were used for categorical variables. p value less than 0.05 ($p<0.05$) was accepted significant.

**RESULTS**

The concentration of smokers HDLs in serum, was significantly ($p<0,001$) lower compared to controls. Triglyceride and Apo B levels of smokers were significantly higher (respectively $p<0,01$ and $p<0,05$) compared to controls but levels of Apo A in the smokers were significantly lower ($p<0,05$) compared to controls (Table 2).

There was no significant difference in the levels of total cholesterol, LDL-C, Lp(a) and hsCRP between the two groups.

The second aim of our study is to measure acute effects of smoking on HDL's antiinflammatory capacity on 25 subjects who gave venous blood sample after 1 times smoking in the morning.

For the second aim of the study blood samples were collected after smoking from twenty five smoking individuals in order to determine the acute effect of smoking.

Before smoking, smokers' total cholesterol, HDL-C, LDL-C, Lp (a), Apo A and hsCRP levels, Apo B levels, did not show a statistically different significance after smoking ($p>0,05$) but before smoking, smokers' TG levels were statistically significant from after smoking ($p<0.001$) (Table 3).

Antiinflammatory properties of HDL-C against LDL-C oxidation was measured by dichlorofluorescein (DCF) assay. Our results indicate that controls and smokers proinflammatory HDL percentage were 10%

**Table 2.** Serum lipid parameters in control and smoker groups (mg/dl)

| Parameters | Control (n=40) Median (25%;75%) | Smokers (n=40) Median (25%;75%) | p value |
|---|---|---|---|
| Total-C | 153.00 (137.25;182.25) | 156.00 (132.50;191.50) | p=0,675 |
| Apo A | 159.40 (141.77;174.05) | 142.35 (126.35;162.90) | p=0.020 |
| Triglyceride | 73.00 (56.25;98.50) | 102.00 (65.25;135.00) | p=0,018 |
| HDL-C | 57.00 (50.00;64.75) | 46.50 (41.00;56.50) | p= 0,001 |
| LDL-C | 84.00 (66.75;116.25) | 94.50 (74.25;119.75) | p=0,133 |
| Apo B | 65.10 (55.52;80.87) | 78.10 (61.22;92.27) | p= 0,036 |
| Lp(a) | 17.35 (10.65;25.10) | 17.65(12.45;24.55) | p=0,791 |
| hsCRP | 0.65 (0.40;1.37) | 0.70 (0.40;1.55) | p=0,946 |

*Mann-Whitney U

**Table 3.** Serum lipid parameters in before smoking and after smoking groups (mg/dl)

| Parameters | Before smoking (n=25) Median (25%;75%) | After smoking (n=25) Median (25%;75%) | p value |
|---|---|---|---|
| Total-C | 156.00 (133.00;191.00) | 154.00 (138.00;191.50) | p=0,951 |
| HDL-C | 47.00 (42.00;58.00) | 49.00 (40.50;59.50) | p=0,393 |
| LDL-C | 94.00 (81.50;118.50) | 90.00 (82.00;115.50) | p=0,343 |
| Triglyceride | 88.00 (69.50;130.00) | 107.00 (76.50;167.00) | p=0,002 |
| Apo A | 138.40 (123.00;152.65) | 137.20 (124.80;151.95) | p=0,898 |
| Apo B | 75.10 (61.85;92.95) | 76.00 (62.60;93.70) | p=0,637 |
| Lp(a) | 17.60 (11.15;24.30) | 17.90 (10.90;26.90) | p=0,248 |
| hsCRP | 0.70 (0.35;1.45) | 0.70 (0.30;1.45) | p=1,000 |

*Mann-Whitney U

and 80% respectively indicating smoking increases proinflammatory HDL levels. Also there were significant differences in the smokers HDL score 1,03(1.00;1.08) compared to controls 0.95(0.90;0.98) (Fig 1 and Table 4A) (p<0.001).

Before smoking and after smoking groups' proinflammatory HDL percentages were 48% and 68% respectively, indicating no significant differences between before smoking group HDL score 1.01(0.98;1.03) and after smoking group HDL score 0.99 (0.96;1.02) (Fig 2 and Table 4B) (p>0.05).

**Table 4.** HDL Score (FU) levels of groups

**A. Effects of smoking on HDL score**

| Groups | 25% | Median | %75 | p value |
|---|---|---|---|---|
| Control (n=40) | 0.90 | 0.95 | 0.98 | |
| Smokers (n=40) | 1.00 | 1.03[a] | 1.08 | p<0,001 |

**B. Acute effects of smoking on HDL score**

| Groups | 25% | Median | %75 | p value |
|---|---|---|---|---|
| Before smoking (n=25) | 0.98 | 1.01 | 1.03 | |
| After smoking (n=25) | 0.96 | 0.99[a] | 1.02 | p=0.078 |

**Table 4A a:** significant difference from control (P<0.001) HDL score (FU)>1 proinflammatory HDL; HDL score (FU)<1 antiinflammatory HDL

**Table 4B a:** no significant difference from before smoking group (P>0.05) HDL score (FU)>1 proinflammatory HDL; HDL score (FU)<1 antiinflammatory HDL

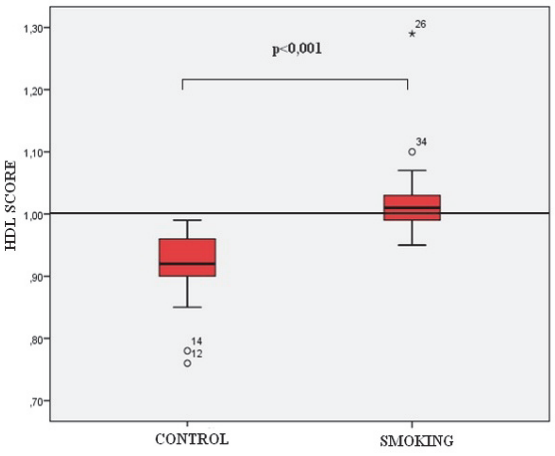


**Figure 1.** HDL Score (FU) levels of control and smoking groups

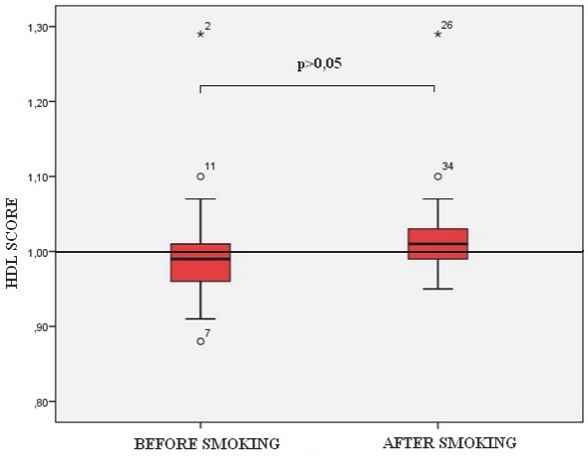


**Figure 2.** HDL Score (FU) levels of smoking groups

## DISCUSSION

In this study, we have aimed to study and evaluate of lipoprotein levels and HDL–C antiinflammatory characteristics between young smokers and healthy nonsmokers. Also, we aimed to study acute effects of smoking in these smokers. In recent studies it's showed that HDLs functions is more important than its quantities (14). It has recently been demonstrated that HDL can decrease oxidative damage to LDL during lipid peroxidation of LDL exposed to endothelial cells. HDL is a modulator of systemic inflammation by promoting it's reverse cholesterol transport. (19).

But, in the presence of systemic inflammation, antioxidant enzymes can be inactivated and HDL can accumulate oxidized lipids and proteins that make it proinflammatory (20). Different factors effect HDLs preventive function against oxidized LDL (14-21). Smoking is known as one of these factors.

In different studies it was shown that different illness which are directly associated with inflammation or cause an inflammatory state increased unfunctional HDL levels (22). In our study proinflammatory HDL was increased among smokers compared to controls (p<0,001) (Fig 1) although their HDLs were in normal range (Table 2) Because smoking produces free oxygen radicals in our body. An excess of free oxygen radicals production due to lack of antioxidants, may increase the risk of coronary artery disease (2). On the other hand there were no significant differences between before smoking and after smoking groups proinflammatory HDL scores (Fig 2) although one puff of a 30 mL cigarette contains 70 billion particles rich in free radicals that cause lipid peroxidation (23). This result may be explained by different styles of smoking. So uncompletely aspiration of cigarette smoke into lungs may not cause lipid peroxidation or not influence proinflamatuar state of HDLs.

Also in our study group, both chronic and acute smoking increased TG levels compared to control. Increased TG levels observed in our study possibly results from nicotine effects. Because nicotine stimulates the secretion of catecholamines as well as other hormones such as cortisol and growth hormone, leading to an increased serum concentration of free fatty acids which stimulates hepatic secretion of very low density lipoproptein and TG (24). Triglyceride metabolism is regulated by the action of lipoprotein lipase (LPL), an enzyme also affected by smoking (23).

Furthermore, apolipoprotein B, the major protein constituent of LDL, may provide

more information on coronary heart risk than LDL-cholesterol alone (2-25). In our study smokers had significantly higher level of Apo B.

Higher level of Apo B is believed to be releated to the risk of premature CAD (26). In these young smokers high Apo B levels may sign long before the onset of symptom of CAD without they have no symptomatic evidence of CAD.

On the other hand in our study HDL levels were in normal range in both smokers and non-smokers but Apo A levels were decreased in smokers (Table 2) These results may be explained by duration of smoking in smokers. Meenakshisundaram et all. showed that in moderate smokers only Apo A was significantly decreased without significant reduction in HDL level as seen in our study (12). Apo A association was stronger than HDL in only heavy smokers. Also smokers low Apo A levels have been associated with significant atherosclerotic changes in patients undergoing coronary angiograph (12). Beside these low Apo A levels, the thyrosine residius in Apo A may be changed by myeloperoxidase which is a determinant of inflammatuar state.

So, Apo A levels and functions may decrease as a result of proinflammatory HDL against LDL oxidation and unability of HDL to promote cholesterol efflux by the ATP-binding cassette transporter A-1 pathway. Also Harmer and et al. showed that smokers have enhanced myeloperoxidase-dependent plasma oxidation (27). Decreasing of smokers Apo A levels in our study may contribute changing normal HDLs to proinflammatuar HDLs.

As a result, smokers' triglyceride, Apo B levels were higher, HDL-C and Apo A levels were lower compared to nonsmokers. Also HDL's proinflammatory charecteristics which indicate an inflammatory state, were higher in smokers.

Lots of smokers had lost their antioxidant capacity of HDL against LDL-oxidation. In this regard chronic smoking is a cause of proinflamatuar HDL levels and proinflamatuar HDL levels may be marker in assesment of cardiac risk and atherosclerosis progress among smokers. Also quality of HDL-C is as important as it's quantity in evaluation of cardiac risk.

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