ORIGINAL ARTICLE

HDL and Apolipoprotein A1 concentrations as markers of cholesterol efflux in middle-aged women: interaction with smoking

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Abstract

OBJECTIVES: It has been demonstrated that the deleterious effect of smoking on the cardiovascular system is mediated through a decrease in protective HDL cholesterol. In addition, women are more sensitive to the negative effects of smoking, although the exact mechanism underlying this phenomenon is currently unknown. In this study, we evaluated whether smoking habits could modify the association of HDL cholesterol and apolipoprotein A1 (ApoA1) with reverse cholesterol transport (RCT), as measured by cholesterol efflux (CHE), in middle-aged women.

DESIGN: The study group consisted of 39 healthy middle-aged women, 21 non-smokers (age 51.8±2.5 years, BMI 25.1±2.8 kg/m^2) and 18 smokers (age 50.5±3.2 years, BMI 24.8±3.5 kg/m^2). In addition to all traditional cardiovascular risk factors, CHE from macrophages, labelled during a 48-hour incubation in a medium containing [^{14}C] cholesterol, to plasma acceptors in study subjects was established as a marker of reverse cholesterol transport.

RESULTS: CHE was significantly higher in non-smokers than in smokers (14.22±1.75% vs. 13.17±1.33%; $p<0.05$). Smoking habit had no effect on the association of HDL with ApoA1 or HDL with CHE. However, in contrast to the strong association of ApoA1 with CHE in non-smokers (r=0.62; $p<0.01$), no such strong association was found in smokers (r=0.38; n.s.).

MAIN FINDINGS AND CONCLUSION: Based on our results, smoking can alter ApoA1-mediated reverse cholesterol transport in women.

Abbreviations:

ApoA1	- Apolipoprotein A1
ABCA1	- adenosine triphosphate binding cassette transporter A1
ABCG1	- adenosine triphosphate binding cassette transporter G1
SR-BI	- scavenger receptor B1
LCAT	- lecithin:cholesterol acyl transferase
CETP	- cholesterol-ester transfer protein
PLTP	- phospholipids transfer protein
NEFA	- non-esterified fatty acid
RCT	- reverse cholesterol transport
CHE	- cholesterol efflux

INTRODUCTION

Epidemiological and interventional studies have clearly established the inverse association between plasma HDL levels (Miller *et al.* 1977) and atherosclerosis. As recently suggested (Khera AV *et al.* 2011), the centripetal return of excess cholesterol from the periphery to the liver via the reverse cholesterol transport pathway is the best-accepted protective effect of HDL in the prevention of atherosclerosis development. The initial step, which is the removal of cellular cholesterol, is made possible almost entirely through interaction between cellular receptors (mainly ABCA1 and SR-BI) and plasma acceptors (HDL particles and their precursors). Approximately 65 % of HDL protein mass is represented by ApoAI. HDL particles have been shown to undergo a loss of function in several pathophysiological conditions (Tan *et al.* 2011, de la Llera Moya *et al.* 2012, Farbstein & Levy, 2012). It has been demonstrated that the deleterious effect of smoking on the cardiovascular system is also mediated through alterations of the function of HDL particles and that women are more sensitive to the negative effects of smoking (Huxley *et al.* 2011).

The data from epidemiological studies have clearly shown that smoking negatively affects concentrations of lipoproteins (i.e., smokers have higher serum concentrations of cholesterol, triglycerides, and low-density lipoprotein cholesterol and lower serum concentrations of high density lipoprotein cholesterol) (Craig *et al.* 1989, Richard *et al.* 1997). Nevertheless, the contribution of smoking to the pathology of atherosclerosis cannot be fully explained by its effect on lipoprotein levels. Ueyama *et al.* (1997) demonstrated that the modification of HDL by cigarette smoke leads to a decrease in cholesterol efflux elicited by these particles in macrophages, apparently due to impaired re-esterification.

In previous studies, we (Kralova Lesna *et al.* 2008 and 2009) and others (Khera *et al.* 2011) have shown that the measurement of HDL and ApoA1 is an ambiguous indicator of the complex process of RCT. In the present study, we evaluated whether smoking habits could alter the cholesterol efflux capacity of HDL particles and ApoA1 in middle-aged women.

METHODS

For the purpose of this study, we analysed the data from 21 non-smoking (age 51.8±2.5 years) and 18 smoking (50.5±3.2 years) middle-aged women previously examined for the effect of menopausal transition on cardiovascular risk. Subjects with metabolic diseases (diabetes mellitus, thyroid gland dysfunction), including metabolic syndrome, or known inflammatory disease were excluded. To exclude metabolic syndrome, we used several already established international criteria from our previous work (Lejskova *et al.* 2011). Body mass index was calculated as weight in kg divided by squared height in meters.

For the purpose of this study, based on our preliminary data and with the goal of detecting differences in cholesterol efflux, we prospectively defined non-smoking status as at least 1 year of abstinence smoking, whereas smoking status was defined as smoking regularly more than 10 cigarettes/day for at least 3 months before the study began. Study subjects were carefully instructed, and they then filled out a questionnaire focused on cardiovascular risk factors including smoking. The study was approved by the Local Ethics Committee and conducted in accordance with the Helsinki Declaration. All participants provided written informed consent prior to enrolment into the study.

Biochemical data

Blood samples were drawn after overnight fasting. Serum total cholesterol and triglycerides were measured using the fully automated (HITACHI 911 Auto Analyzer, Japan) enzymatic method (reagents from Hoffmann, La Roche, Basel, Switzerland). HDL-cholesterol was determined using the same method after precipitation of serum lipoproteins with sodium phosphotungstate and magnesium chloride kits. Serum LDL cholesterol was measured using an automated method with direct determination using an LDL-C plus kit from Hoffmann-LaRoche (Basel, Switzerland). ApoA1 and ApoB concentrations were measured by the immunoturbidimetric assay (Orion Diagnostica, Espoo, Finland). All biochemical analyses and CHE measurements were simultaneously performed at the end of the study to eliminate inter-assay variation.

RCT was measured using human macrophages prelabelled with medium containing [^{14}C] cholesterol as described in detail recently (Kralova Lesna *et al.* 2008). Briefly, THP-1 human monocytes (human monocytic leukaemia cells, ECACC 88087201), were maintained in RPMI 1640 medium containing 10% foetal bovine serum, 2 mM glutamine and 1% penicillin/streptomycin (PAA Laboratoires) at 37 °C with 5% CO_2. THP-1 monocytes were seeded into 24-well plates in the presence of phorbol 12-myristate 13-acetate (100 ng/ml) (Sigma-Aldrich) for 72 h to induce differentiation into macrophages. These macrophages were subsequently labelled during a 48-hr incubation in medium containing [^{14}C] cholesterol (specific activity 0.2 μCi/ml) (PerkinElmer Life Sciences, Inc.). To measure cholesterol efflux, cells were incubated for 240 minutes with RPMI containing 5% plasma from the study subjects. Cholesterol efflux (%) was expressed as the radioactivity of the efflux media divided by the total radioactivity of the sample (media plus lysed cells). Each plasma sample was analysed in triplicate, and the data presented are means of the triplicates.

All results are expressed as the mean ± standard deviation (SD). The differences between groups were evaluated using a non-paired t-test. The relationship between changes of CHE and changes of lipoprotein parameters was analysed by simple linear regression. The signifi-

cance of the difference between two correlation coefficients was assessed using Fisher transformation.

RESULTS

As shown in Table 1, significantly higher HDL-C was detected in non-smokers than in smokers (1.97±0.36 vs. 1.53±0.33 mmol/l; $p<0.05$). No significant differences in other lipid parameters or body mass index were observed between non-smokers and smokers. Smoking habit had no effect on the correlation of HDL with ApoA1 (correlation coefficient 0.71 in non-smokers and 0.69 in smokers, data not shown). CHE was significantly higher in the non-smokers than in smokers (14.22±1.75% vs. 13.17±1.33%; $p<0.05$). Significant correlations were found for HDL cholesterol with CHE in both groups (non-smokers: r=0.61; $p<0.01$, smokers: r=0.71; $p<0.01$), but there were no significant differences between the correlations within the respective groups (Figure 1). However, the correlation of ApoA1 with CHE differed between smokers and non-smokers. Whereas in non-smokers, this correlation was again strong (r=0.62; $p<0.01$), in smokers, it was substantially weaker (r=0.38, p=n.s.) (Figure 2).

Tab. 1. Anthropometric parameters, concentration of lipids, lipoproteins and the rate of cholesterol efflux to plasma in study subjects.

	Non-smokers (n=21)	Smokers (n=18)
Age (years)	51.8±2.5	50.5±3.2
Body mass index (kg/m^2)	25.1±2.8	24.8±3.5
No. of cigarettes/day	0	15.3±3.2
Total cholesterol (mmol/L)	5.57±0.93	5.57±1.33
Triglycerides (mmol/L)	1.12±0.49	1.84±2.20
LDL cholesterol (mmol/L)	3.22±0.81	3.18±0.86
HDL cholesterol (mmol/L)	1.97±0.36	1.53±0.33**
Apo B(g/L)	1.02±0.33	1.10±0.39
Apo A1 (g/L)	1.71±0.27	1.59±0.29
CHE (%)	14.22±1.75	13.17±1.33*

*$p<0.05$; **$p<0.01$ (non-smokers vs. smokers, nonpaired t-test)

DISCUSSION

In this study, we determined that smoking could alter the ApoA1-mediated effect on reverse cholesterol efflux in middle-aged women. Our data argue that ApoA1 concentration is therefore not a reliable indicator of reverse cholesterol transport in smokers. As expected, the results of this study also indicate that the plasma of smokers evokes lower cholesterol efflux compared to non-smokers.

To the best of our knowledge, this work is the first study to examine the effect of smoking on the relationship between HDL and ApoA1 concentrations and the capacity of plasma to evoke cholesterol efflux. Nevertheless, our results are consistent with the only published study to focus on the effect of cigarette smoke on cholesterol efflux from macrophages that used purified HDL particles modified by direct contact with smoke extracts (Ueyama *et al.* 1997). The authors described the functional impairment of HDL particles exposed to smoke extract, an effect attributed to the increase in lipid peroxidation.

In the present study, we used whole diluted plasma and therefore cannot distinguish between the multiple

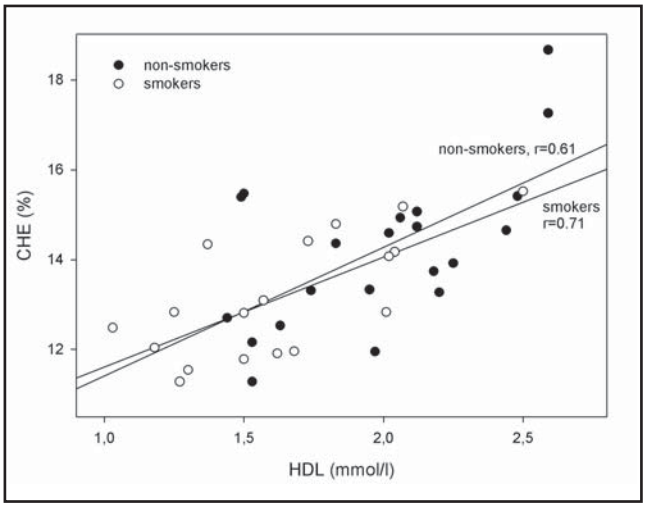

Fig. 1. The relationship between the HDL-C and cholesterol efflux (CHE) in smokers and non-smokers. Correlations: non-smokers r=0.61, $p<0.01$; smokers r=0.71, $p<0.01$, probability of the same strength of correlations $p<0.61$.

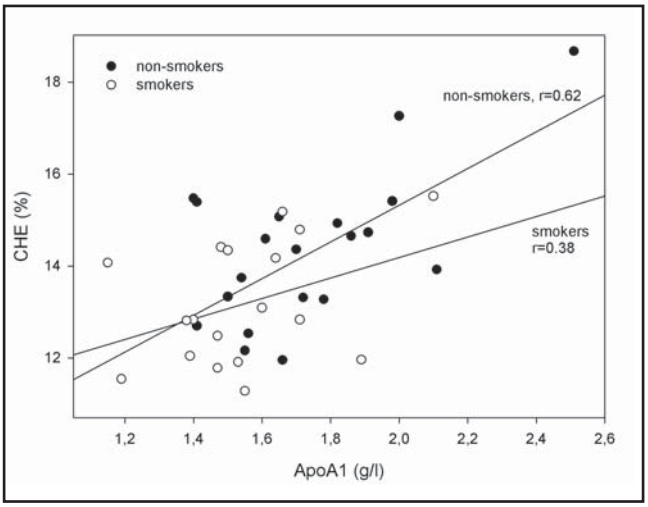

Fig. 2. The relationship between the ApoA1 and cholesterol efflux (CHE) in smokers and non-smokers. Correlations: non-smokers r=0.62, $p<0.01$; smokers r=0.38, p=n.s.

steps of cholesterol transport. As we are well aware of the possibility of modification of HDL subfractions during isolation, we preferred to use whole plasma. The other advantage of this approach is that this appears to be the best approximation of an *in vivo* situation. We decided to use modified macrophages because they play a crucial role in the pathogenesis of atherosclerosis. This cell model has been commonly used in similar studies. For example, the presence of receptors (ABCA1, ABCG1 and SR-BI) involved in cholesterol efflux, which is the key step in RCT, was demonstrated using modified macrophages (Beroughi *et al.* 2006; Uehara *et al.* 2007).

The lower HDL concentrations in smokers are consistent with results from the Framingham study (Garrison *et al.* 1978). In contrast to already published results (reviewed by Chelland Campbel *et al.* 2008), we did not detect any significant differences in LDL and TG levels between smokers and non-smokers. This could be due to the relatively small number of subjects and the resulting decreased statistical power to detect such differences. In addition, women with metabolic syndrome were not included, which might obscure the effect of smoking on triglycerides even more.

The evidence linking cigarette smoke exposure with cardiovascular disease was demonstrated by numerous epidemiological studies, as well as by the positive effect of smoking cessation in the secondary prevention of CVD (Gordon *et al.* 1989), but the exact mechanisms responsible for this association have not been fully elucidated. Clinical and experimental observation have demonstrated the effect of smoking on several known risk factors for atherosclerosis (i.e., vasomotor and endothelial haemostatic dysfunction (Mayhan & Sharpe, 1996, reviewed Cacciola *et al.* 2007), inflammation (reviewed in Arnson *et al.* 2010), and modification of lipid profile (Chelland Campbell *et al.* 2008)).

The possibility that the ability of HDL particles and their precursors to induce CHE could be altered *in vivo* due to various conditions was recently shown. Tan *et al.* (2011) demonstrated that in type 2 diabetic patients, there is an impairment in the cholesterol efflux to small HDL particles, which are considered to be efficient acceptors of cholesterol with cholesterol efflux. This finding raises the possibility that the small HDL particles might be dysfunctional due to metabolic alteration, as non-enzymatic glycosylation of HDL has been shown to impair the capacity to support cholesterol efflux (Hoang *et al.* 2007). A recent study (de la Llera Moya *et al.* 2012) has shown an impairment in both SR-BI- and ABCA1-mediated cholesterol efflux in artificially induced inflammation in humans. It seems plausible that cholesterol efflux is a vulnerable process due to the modification of HDL particles (and their precursors).

The main limitation of the study is its small number of subjects. Therefore, it needs to be established whether these results are valid for men and premenopausal women as well. Another limitation of the study is its cross-sectional design. Therefore, cause and effect relationships are less obvious, and our study is rather hypothesis-generating. Longitudinal interventional studies focusing on the effect of smoking cessation on ApoA1 and reverse cholesterol transport should confirm these results.

In conclusion, we have demonstrated that cholesterol efflux, widely accepted as the most important step in reverse cholesterol transport, is lower in smoking compared to non-smoking women. Our results further indicate that the impairment of this process is mainly based on the modification of ApoA1, as the association of ApoA1 with CHE is almost entirely lost in smokers.

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