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ASSOCIATIONS BETWEEN SMOKING STATUS, LIFESTYLE AND LIPOPROTEIN SUBCLASSES

ABSTRACT

Background: The relationship between cigarette smoking and cardiovascular disease is well-established, yet the underlying mechanisms remain unclear. Although smokers have a more atherogenic lipid profile, this may be mediated by other lifestyle-related factors. Analysis of lipoprotein subclasses using nuclear magnetic resonance spectroscopy (NMR) may improve characterisation of lipoprotein abnormalities.

Objective: We used NMR spectroscopy to investigate the relationships between smoking status, lifestyle-related risk factors and lipoproteins in a contemporary cohort.

Methods: A total of 612 participants (360 women) aged 40-69 years at baseline (1990-1994) enrolled in the Melbourne Collaborative Cohort Study had plasma lipoproteins measured using NMR. Data were analysed separately by sex.

Results: After adjusting for lifestyle-related risk factors including alcohol and dietary intake, physical activity and weight, mean total low-density lipoprotein (LDL) particle concentration was higher for female smokers than non-smokers. Both medium- and small-LDL particle concentrations contributed to this difference. Total high-density lipoprotein (HDL) and large-HDL particle concentrations were lower for female smokers than non-smokers. The proportion with low HDL particle number was higher for female smokers than non-smokers. For men, there were few smoking-related differences in lipoprotein measures.

Conclusions: Female smokers have a more atherogenic lipoprotein profile than non-smokers. This difference is independent of other lifestyle-related risk factors. Lipoprotein profiles did not differ greatly between male smokers and non-smokers.

Key words: Atherosclerosis, smoking, lipoproteins, lifestyle, spectroscopy

Abbreviations: CVD **=** cardiovascular disease

LDL = low-density lipoprotein

HDL = high-density lipoprotein

VLDL = very low density lipoprotein

IDL = intermediate density lipoprotein

NMR = nuclear magnetic resonance

LDL-C = LDL-cholesterol

CHD = coronary heart disease

WHR = waist-hip ratio

BMI = body mass index

HDL-C = HDL-cholesterol

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Cigarette smoking is a major risk factor for cardiovascular disease (CVD).[1] Although underlying mechanisms are not fully elucidated, many studies demonstrate an atherogenic lipid profile for smokers, including higher total cholesterol, low-density lipoprotein (LDL)-cholesterol and triglycerides, and lower (usually vasoprotective) high-density lipoprotein (HDL)-cholesterol levels.[1-4] It is unclear as to the extent by which relationships between smoking and lipoproteins are influenced by other lifestyle-related risk factors, such as poor nutrition, inactivity and adiposity. [5] Further, because of differences in lipoprotein composition, patients with the same lipid concentrations may have substantially different CVD risk, particularly in the context of comorbidities such as diabetes. [6] Detailed lipoprotein subclass analysis might therefore increase understanding of the relationship between smoking, other lifestyle-related risk factors, lipoproteins and CVD risk.[6]

There are three major lipoprotein classes: Very Low Density Lipoprotein (VLDL), LDL and HDL. Intermediate Density Lipoprotein (IDL), also known as VLDL remnants, is highly atherogenic, but usually exists at low levels. There are at least three main subclasses, large, medium and small recognised for each of VLDL, LDL and HDL.(7) Traditional methods of measuring lipoprotein subclasses include gradient gel electrophoresis and density-gradient ultracentrifugation, both of which are time-consuming and not clinically available. An accurate, faster and more available approach to their measurement is nuclear magnetic resonance (NMR) spectroscopy. [8] NMR studies have shown that LDL particle concentration is independently associated with and a better predictor of CVD events than LDL-cholesterol (LDL-C) concentrations, [9] and that both small LDL and large VLDL are positively associated with coronary heart disease (CHD), while large HDL are protective. [2, 10]

In the Framingham Offspring Study, NMR has been used to investigate the relationship between smoking, small LDL concentrations and oestrogen receptor alpha gene variation[3] and also to evaluate smoking intensity effects on lipoprotein concentrations for current smokers.[11] No NMR studies have examined differences between non-, former and current smokers, nor controlled

for potential influences of lifestyle-related risk factors. This Australian study investigated such relationships in a contemporary cohort of men and women.

METHODS

Study sample

The prospective Melbourne Collaborative Cohort Study recruited 41 514 subjects aged 27-80 years in 1990-94. Twenty-four percent were southern European migrants, deliberately over-sampled to extend the range of lifestyle factors and genetic variation. Study details have been published elsewhere. [12] Approval from The Cancer Council Victoria's Human Research Ethics Committee and written informed consent from participants were obtained. Baseline examination included face-to-face interviews and questionnaires in the subject's preferred language.

A random sample of 934 fasted subjects who met the eligibility criteria of having 3 ml of stored plasma, and a fasting plasma glucose <7.0 mmol/L (Kodak Ektachem analyzer; Rochester, New York) was selected. Participants were excluded for: subsequent cancer (n=35); history of heart attack, stroke, angina or diabetes (n=71); currently taking lipid-lowering therapy (n=17); unknown menopause status (n=12); missing data on cigarette smoking or covariates (n=2); or missing data on conventional lipids (n=125). Data from 612 participants (360 women and 252 men) were analysed. Separate analyses were undertaken for men and women because of known gender differences in lipids and NMR-determined lipoproteins.[13]

Smoking Status

Smoking status was ascertained from questions modified from the validated Medical Research Council 1986 Respiratory Symptoms Questionnaire.[14] Smokers were defined as people currently smoking ≥7 cigarettes a week, non-smokers as never having smoked and former smokers as having previously smoked but now ceased, or those smoking <7 cigarettes a week. Time since smoking cessation ranged from 1-25 years.

Baseline demographics and cardiovascular risk factors

Country of birth was categorised as: 1) Australia/New Zealand/northern Europe (primarily UK and the Netherlands), or 2) southern Europe (mainly Italy and Greece). Additional covariates for women included oral contraceptive use (never/past and current), hormone replacement therapy (never/past and current) and menopausal status (pre- and post-).

Participants were asked their usual quantity and frequency of alcohol intake and were considered current drinkers if they responded yes to: "Have you ever drunk at least 12 alcoholic drinks in a year (sips don't count)?" Physical activity over the previous six months was based on the number of times per week that exercise was undertaken at a vigorous, less vigorous or walking level only. These data were combined to give an overall score of relative energy expenditure in four categories, based on the Compendium of Physical Activities. [15] Dietary information was collected using a validated, self-administered food frequency questionnaire. [16] Average intake of 121-items over the previous year was used to calculate average daily nutrient intake. Waist-hip ratio (WHR) and body mass index (BMI) were calculated from direct measurements using standard anthropometric methods.(17) The second and third of three blood pressure readings measured (DINAMAP 1846SX) after 5mins supine rest were used in analyses. Metabolic syndrome diagnosis was based on the 2006 International Diabetes Federation criteria.[18]

Conventional lipid measures

Total cholesterol, HDL-cholesterol (HDL-C) and triglyceride levels were measured using methods previously described.(19) LDL-C was calculated using the Friedewald formula. [20]

NMR-measured lipoproteins

VLDL, LDL and HDL subclass particle concentrations and mean particle diameters were measured with an automated NMR spectroscopic assay as previously described(7). CHD risk according to total LDL particle number was defined as follows:[21] Total LDL particle number as

>2000nm/l and 1600-2000 nm/l (both associated with increased risk); Small LDL particle number as >1200nmol/l and 850-1200 nmol/l (associated with increased and borderline CHD risk respectively); and large HDL particle number as <4.0µmol/L (also associated with increased CHD risk). Small LDL (pattern B) was defined as mean LDL diameter <20.5nm.

Statistical Analyses

Stata 9.2 (Stata Corp, College Station, Texas, USA) was used. Linear regression models for lipoprotein subclass measurements analysed the associations with smoking, adjusted for age, country of birth, education, alcohol, physical activity, WHR, BMI, total daily energy, carbohydrate and saturated fat intake. Oral contraceptive use, hormone replacement therapy, and menopausal status were also adjusted for in women. Two-sided p-values are presented and values <0.05 were regarded as significant.

As there was collinearity between total daily energy and carbohydrate intake, the carbohydrate variable was replaced by converting carbohydrate (gm/day) to an energy equivalent and dividing by total daily energy intake. Large and medium VLDL and medium HDL followed skewed distributions. VLDL lipoproteins were therefore log transformed prior to analysis, and medium HDL data was analysed using logistic regression. Analysis of differences in CHD risk between smokers and non-smokers according to lipoprotein particle size and number was performed using univariate logistic regression.

The percentage difference in lipoprotein concentrations for smokers compared with non-smokers was calculated as:

$$\frac{\textit{(mean lipoprotein value in smokers-mean lipoprotein value in non-smokers)}}{\textit{Standard deviation of lipoprotein in non-smokers}} \quad X\ 100$$

RESULTS

Subjects

Baseline characteristics in **Table 1** show differences between males and females in the comparability of smokers and former smokers to non-smokers. For women, current smokers

had a lower mean BMI than non-smokers, and were more likely to be normal weight. Compared with female non-smokers, former smokers were less likely to have been born in a Mediterranean country, to have primary education only, and to be obese. Additionally, female former smokers were more likely to drink alcohol than non-smokers.

For men, current smokers were more likely to be Mediterranean-born, to have primary education only, and to be physically inactive than non-smokers. Compared with male non-smokers, former smokers were older, more likely to be Mediterranean-born and to have primary education only. Male former smokers also had a higher daily alcohol intake, lower carbohydrate intake, lower saturated fat intake, and were more likely to be obese than non-smokers.

Conventional Lipids

Females

As shown in **Table 1**, female smokers had higher triglycerides and total cholesterol/HDL-C ratio, and a lower HDL-C than non-smokers in both univariate analysis and following adjustment for lifestyle-related factors. LDL-C levels were higher in smokers than non-smokers, although these differences were only apparent after multivariate adjustment.

In female former smokers, triglycerides were higher compared with non-smokers ($p=0.02$). HDL-C was higher in former smokers than current smokers ($p=0.02$), and total/HDL-C ratio was lower ($p=0.001$), with similar concentrations of both these lipids in non-smokers and former smokers. All differences were significant after multivariate adjustment.

Males

In men, the only significant smoking-related differences were in total and LDL-cholesterol, which were both less in former smokers than in current smokers (both $p=0.03$) after

multivariate adjustment, with levels similar to those of non-smokers. There were no significant differences in triglycerides between smoking categories.

NMR-measured Lipoproteins

Females

As shown in **Table 2**, no differences were seen in VLDL particle concentrations, but VLDL was larger in smokers compared with non-smokers. Median total LDL particle concentration was also higher in smokers than non-smokers, with both medium and small LDL particle concentration contributing to this difference. The evidence for these findings was compelling, regardless of whether or not lifestyle factors were adjusted for. Median total HDL and large HDL levels were lower in smokers than for non-smokers in both univariate and multivariate analysis.

Median large VLDL levels were higher in former smokers than in non-smokers ($p=0.03$). Total LDL levels were lower in former smokers than current smokers ($p=0.01$), with similar patterns in medium and small LDL concentrations ($p=0.01$ for both). Total HDL particle concentration was increased in former smokers compared with current ($p=<0.001$) and non-smokers ($p=0.02$), with large HDL concentrations higher in former smokers than in current smokers only ($p=0.002$).

Figure 1 shows the percentage difference in crude mean values of NMR-determined lipoprotein measures in females. Compared to non-smokers, current smokers had higher values for total LDL and small LDL of 49% and 37% respectively, and lower values of total HDL and large HDL of 41% and 33% respectively.

Males

There were few smoking-related differences in lipoprotein particle concentration or size in males **(Table 2)**. After multivariate analysis, large LDL concentration and LDL size were higher in current smokers compared with non-smokers. Small VLDL concentration was lower in non-smokers than in smokers ($p=0.01$), and VLDL size was increased ($p=0.04$).

Proportions at increased risk of CHD

The proportion of men and women with increased CHD risk NMR profiles is shown in **Table 3**. Based on total LDL particle levels, 22% of female smokers were at increased CHD risk compared with 4% of female non-smokers ($p<0.001$). A greater proportion of female smokers had small LDL particle number >1200nmol/L and low levels of large HDL than non-smokers.

For men, there were no statistically significant differences in the proportions of those with NMR profiles associated with increased CHD, although there was a suggestive trend towards more smokers than non-smokers with mean LDL particle size ≤20.5nm ($p=0.06$); an opposite pattern to that seen in women.

DISCUSSION

We demonstrate that female smokers have a more atherogenic lipoprotein profile than non-smokers, although this profile is less adverse in ex-smokers. Differences are seen in both conventional lipid concentrations and in NMR-determined lipoprotein profiles, and are independent of lifestyle-related factors. There were few smoking-related changes in lipoproteins for males.

Current smokers

For female smokers, higher total triglycerides, LDL-C and total/HDL levels and lower HDL-C have been previously reported. [3-4] Detailed NMR-determined lipoprotein analyses show that females had a higher total LDL particle concentration than non-smokers, contributed to by both medium and small LDL. Female smokers also had lower total HDL particle concentrations than non-smokers, primarily due to a decrease in large HDL particles. Small, dense LDL particles are more atherogenic than large, buoyant LDL particles, [8] and large HDL particles appears to be associated with greater vasoprotection than small HDL particles. [10] Therefore, our findings point to a more atherogenic lipoprotein profile for female smokers compared with non-smokers. Further, while the percentage differences in the median levels of these lipoprotein subclasses for

female smokers are small, they still lead to large differences in the proportions of those with higher values. For example, the proportion of female smokers with increased-risk small LDL was 33% higher than that of non-smokers.

Our results are similar to NMR sub-study in The Framingham Offspring Study.[3] An earlier British cross-sectional study using gradient gel electrophoresis also found that lower HDL-C levels for female smokers related to lower concentrations of large HDL subfractions. [2]

Former Smokers

Female former smokers had higher HDL-C and lower total/HDL-C levels than current smokers, and similar levels to non-smokers. Other studies combining men and women have also shown that smoking cessation is associated with a return of HDL-C to levels seen for non-smokers. [22] Plasma HDL-C concentrations rise within 2-8 weeks following smoking cessation, [22] but temporal associations could not be examined in our study as time since smoking cessation was measured in years only.

Relative to current smokers, female former smokers also had higher levels of total HDL and large HDL particle concentrations. Additionally, both total and medium LDL particle concentrations were lower than those of current smokers, suggesting reversibility of smoking effects on these lipoprotein subclasses. No other studies have examined effects of smoking cessation on NMR-derived lipoprotein measures.

Differences between men and women

There were few statistically significant changes in conventional or NMR-determined lipoprotein profiles by smoking categories for men. Other studies have also found that smoking-related lipoprotein changes are less for men than women. [3-4] For example, the PROCAM study reported similar HDL-C and triglyceride concentrations for male and female smokers, but the increases in total and LDL-cholesterol for women were two and almost four-fold (respectively) that of men.[4]

Freeman et al found similar triglyceride levels for male current smokers and non-smokers, although differences existed for women.[2] Additionally, HDL_2 (the larger, more buoyant and cholesterol-rich HDL subclass as measured using analytical ultracentrifugation) was lower for both men and women, but only reached statistical significance for women.[2] These differences may relate to underlying sex differences in smoking effect mechanisms [2] that may be hormonal in nature. [23]

Lifestyle factors and lipids

Consistent with our findings, other studies have shown that the adverse lipid profile of smokers is independent of potential confounding lifestyle factors. [24-25] We found that smoking-related differences in lipoprotein measures remained significant after adjustment for body weight, activity, alcohol and diet, all of which are known to affect LDL or HDL levels.[26-27] There were few differences in these lifestyle factors between the smoking groups in our cohort. Therefore, our findings may be due to this homogeneity, rather than to any true lack of effect. In our study, female former smokers were more likely to be current alcohol drinkers than were non-smokers, and to also have higher levels of HDL-C, HDL particle concentration and large HDL. Moderate alcohol intake by women has been associated with increased HDL-C in other studies, [28] and also with higher levels of HDL particle concentration and large HDL for older adults in the Cardiovascular Health Study.[29]

Limitations

This study was conducted in Melbourne, Australia. Although these findings may not be applicable to other regions, our results support those in the (USA) Framingham Offspring Study.[3] Generalisability may also be affected by oversampling of southern Europeans. Additionally, smoking and other behavioural risk factors were self-reported. Any resultant misclassification, assuming it was not differential according to smoking status, was likely to bias findings towards null effects.

Estimation of LDL-C from the Friedewald formula may be fallacious when triglyceride levels are >4.5mmol/l. [30] However in the present study, only one female smoker had such a level.

A dose-response between the intensity of smoking and lipids has been seen in other studies.[1] *However, due to the relatively small sample size in our study, we unable to investigate effects according to number of cigarettes smoked/day or other sub-group analysis such as alcohol consumption.* Finally, while the cross-sectional study design means it is not possible to ascertain any causal relationship between smoking and lipoprotein, a significant body of evidence from other studies supports the causal nature of the association. [1-4]

In summary, the smoking-related differences we observed in our study occurred independently of lifestyle factors, indicating that treatment of dyslipidaemia, particularly for female smokers, may require more than adherence to guidelines for activity, diet and alcohol intake. By identifying the specific contribution of lipoprotein subclasses to the adverse lipoprotein profile and subsequent increased CVD risk for smokers, our findings have important implications for the interpretation of results and management of dyslipoproteinaemia.

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