

Secondhand smoke (SHS) exposure is associated with circulating markers of inflammation and endothelial function in adult men and women

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Word count: body 4445, abstract 250, 3 tables.

Abbreviations

SHS secondhand smoke

MI myocardial infarction

CVD cardiovascular disease

CHD coronary heart disease

CI confidence interval

HDLc high density lipoprotein cholesterol

CRP C-reactive protein

IL-6 interleukin-6

IL-18 interleukin-18

VWF von Willebrand Factor

TNF α Tumor necrosis factor-alpha

MMP-9 matrix metalloproteinase-9

BMI body mass index

BP blood pressure

SBP systolic blood pressure

DBP diastolic blood pressure

t-PA tissue plasminogen activator

LR Likelihood Ratio

Acknowledgements:

The British Regional Heart Study is a British Heart Foundation (BHF) Research Group and is supported by BHF programme grant (RG/04/003). Cotinine analyses were funded by BHF project grant PG/06/154/22043. Cytokine analyses were funded by BHF project grant PG/07/048/22395ILI. The British Women's Heart and Health Study is joint-funded by the UK Department of Health and BHF. The views expressed in this publication are those of the authors and not necessarily those of the funding bodies.

Conflict of interest: none declared

Key words secondhand smoke, CVD risk factors, inflammation, epidemiology

Abstract

Aims: Secondhand smoke (SHS) exposure is associated with elevated CHD risks. Yet the pathways through which this may operate have not been investigated in epidemiologic studies with objective SHS exposure measures and a wide range of CHD risk factors associated with active smoking. Therefore we investigate associations between SHS exposure and CHD risk factors, to clarify how SHS exposure may raise risk of CHD.

Methods: Cross-sectional population-based study of 5029 men and women aged 59-80 years from primary care practices in Great Britain. Smoking, behavioural and demographic information was reported in questionnaires; nurses made physical measurements and took blood samples for analysis of serum cotinine and markers of inflammation, hemostasis and endothelial dysfunction.

Results: Active cigarette smokers had lower albumin and higher triglycerides, CRP, IL-6, white cell count, fibrinogen, blood viscosity, factor VIII, VWF and t-PA than non-smokers. Among non-smokers, serum cotinine levels were independently positively associated with CRP, fibrinogen, factor VIII, VWF and t-PA and inversely associated with albumin, after adjustment for age, gender, social and behavioural factors. The differences in CRP, fibrinogen and albumin between cotinine ≤ 0.05 and >0.7 ng/ml were one-third to one half the size of differences between cotinine ≤ 0.05 ng/ml and current smokers, but were of similar magnitude for VWF and t-PA.

Conclusions: Endothelial, inflammatory and haemostatic markers related to CHD risk showed independent associations with SHS exposure in the same direction as those for active smoking. Results aid understanding of the associations between SHS exposure and elevated CHD risks.

Introduction

Secondhand smoke (SHS) is associated with elevated risks of coronary heart disease (CHD) (1-3) and stroke(4). Epidemiologic studies based on self-reported exposure to SHS have reported a 1.2- to 1.3- fold increase in risk of CHD, independent of established CHD risk factors(2;3); whilst a study using serum cotinine as a marker of overall SHS exposure reported slightly higher hazard ratios, around 1.4(5). In both types of study, the risks associated with high SHS exposure were very similar to risks from light active smoking, despite the much greater exposure to tobacco smoke in active smoking(1;5;6). However, the mechanisms by which SHS elevates CHD risk remain uncertain. Several possible pathways are indicated by experimental studies in adults; acute SHS exposure has been shown to increase platelet activity (7;8), lower HDL-cholesterol (HDLc) levels (9), elevate homocysteine levels (10),or increase circulating levels of inflammatory and hemostatic markers which are themselves associated with CHD risk, such as white cell count (11). Experimental data also suggest that SHS may cause endothelial damage, increasing endothelial cell turnover (7) and causing a similar degree of endothelial dysfunction to that observed in active smokers (12). Active cigarette smoking influences all these pathways (13-16).

Whilst most evidence about how SHS affects cardiovascular risk factors comes from short term laboratory studies(3), evidence about the relation of SHS exposure to the risk pathways at the population level is limited. Most epidemiologic studies of SHS exposure and CHD risk factors rely on self-report (17). To date cotinine, a quantitative marker of recent tobacco exposure(18), has been used to measure SHS exposure in relation to CHD risk factors in only two population-based studies, the British Regional Heart Study and the third National Health and Nutrition Examination Survey (NHANES III) (5;19;20). Population studies indicate that SHS exposure (either self-report or cotinine level) is associated with significantly higher systolic and diastolic blood pressure (SBP and DBP), BMI (5;20) and homocysteine levels(17;19), although

associations with lipids (19-21), white blood cell count, C-reactive protein (CRP) and fibrinogen(17;19;22) are less consistent.

In order to examine potential pathways linking SHS exposure to CHD risk, we examine the epidemiological associations between SHS exposure, assessed by serum cotinine, with a wide range of activation markers of inflammation and hemostasis that existing data suggest could be part of the response to SHS exposure and are implicated in CHD risk in older men and women. Analyses examine the effect of adjustment for important confounders and set the associations between SHS exposure and risk factors in the context of associations observed in active smokers. We exclude from analyses ex-smokers who quit recently (to avoid carry over effects), participants taking warfarin (which would affect levels of coagulation factors) and participants with a history of MI, diabetes or stroke, to avoid reverse causality.

METHODS

Study design

Two parallel prospective studies of 60-79 year olds: 4252 men surveyed in 1998-2000 and 4286 women surveyed in 1999-2001. Participants were from a single General Practice in 24 British towns, 77% response rate (men)(23) and 60% (women)(24). Near identical protocols for data collection were used. Participants completed detailed questionnaires including health behaviour data. Nurses made physical measurements and collected fasting venous blood samples (see supplementary data). All participants provided written informed consent to the investigation and ethical approval was provided by all relevant local Research Ethics Committees.

Laboratory assays

Liquid chromatography tandem mass spectrometry was used to assay cotinine in serum samples of non-smokers, the unit of quantification was 0.1 ng/mL and the lower limit of detection was

0.02 ng/mL. Cotinine values at the limit of quantification (0.1) were assigned a value of 0.05ng/ml. Further details, including assays for blood lipids and metabolic markers, inflammatory and hemostatic markers are described in supplementary data.

Classification of smokers and non-smokers

Non-smokers reported no current cigarette, cigar or pipe smoking or any smoking in the past 5 years, and had serum cotinine ≤ 15 ng/mL, consistent with other literature (25). 99% of self-reported non-smokers with cotinine data had levels < 15 ng/mL, 76 non-smokers with cotinine > 15 ng/mL were recoded as smokers of 1-9 cigarettes/day. No participants reported taking nicotine replacement therapy (British National Formulary code 4.10) (26).

Statistical methods

Serum cotinine was highly positively skewed and therefore analyzed as categories, the highest exposure group was chosen because cotinine > 0.7 ng/mL is reportedly associated with elevated CHD risks(5), the lowest group had undetectable exposure and participants with intermediate exposure were split into two equal groups. Cotinine was also natural log transformed to base 2 to display the effect of doubling in cotinine level. Means or proportions of behavioural and demographic factors selected *a priori* were calculated for groups of non-smokers (defined by cotinine level) and active smokers (defined by cigarettes/day). *P* values for linear trends in cotinine levels in non-smokers were obtained using linear or logistic regression analyses of each of the behavioural or demographic variables, with $\log_2[\text{cotinine}]$ as a predictor, adjusted for age, gender and region of residence. CVD markers were examined: skewed variables were natural log-transformed and adjusted for time of measurement if they showed significant diurnal variation. BP, BMI and waist circumference were also adjusted for intra-observer variation. No diurnal or seasonal (October-March vs April-September) variation in cotinine levels was observed. The percentage change between the mean CVD marker levels for non-smokers in the lowest

compared to highest cotinine categories were calculated as $[(\text{mean} \leq 0.05 \text{ ng/mL}) - (\text{mean } 0.71 - 15 \text{ ng/mL})] / \text{mean} \leq 0.05 \text{ ng/mL}$. Percentage differences were also calculated comparing non-smokers with $\leq 0.05 \text{ ng/mL}$ cotinine to smokers (1-9 cigarettes/day).

Linear regression models were used to estimate the linear associations between each inflammatory, endothelial or hemostatic marker (response variable) and $\log_2[\text{cotinine}]$, so the beta coefficients represent the risk factor difference associated with a doubling in cotinine concentration. Models were first adjusted for age, gender and region of residence, and next for physical inactivity, light alcohol drinking, smoking history (time since quitting smoking or never-smoking) and social class as categorical variables and BMI as a continuous variable. Interaction terms between BMI and gender were fitted for the inflammatory markers where interactions were evident (likelihood ratio (LR) test $P < 0.05$). Finally, adjustments were made for HDLc, triglycerides and SBP as continuous variables. Further, logistic regression models contrasted cotinine ≤ 0.05 with $> 0.7 \text{ ng/mL}$, because cotinine $> 0.7 \text{ ng/mL}$ was reportedly associated with elevated CHD risks(5). Age and gender were examined as modifiers of the cotinine-CVD marker associations. Interactions were tested using LR tests. As sensitivity analyses, models were restricted to (i) never-smokers and (ii) participants with cotinine $\leq 9.5 \text{ ng/mL}$.

RESULTS

Among 8152 participants (4267 women, 4245 men) with questionnaire data on cigarette smoking, 90% (n=7375) had cotinine assays. The analysis sample excluded (i) n=251 (3%) ex-smokers who quit < 5 years previously, (ii) 162 taking warfarin (iii) 1472 with a history (self-report or record in GP notes) of MI, diabetes or stroke, (iv) 733 active cigarette smokers. The analysis sample comprised 4757 non-smokers (of which 52% never smoked). For comparison, descriptive analyses included 272 smokers of 1-9 cigarettes/day. Of 4757 non-smokers, 38% had undetectable cotinine ($\leq 0.05 \text{ ng/mL}$) and the remainder were divided into 3 groups (Table 1).

The characteristics of active smokers and non-smokers with different levels of SHS exposure are shown in Table 1. Geometric mean cotinine was much higher in smokers than non-smokers; 100.58 ng/mL vs 0.15 ng/mL, $P < 0.001$. Active smokers tended to be from manual social groups, resident in the north of the UK, and physically inactive. Non-smokers with higher compared to lower serum cotinine levels (greater SHS exposure), were also from manual social class groups, resident in the north of the UK, physically inactive and were younger, male, ex-smokers rather than never-smokers.

Table 2 presents adjusted mean values of CVD risk markers for active smokers and non-smokers with different levels of SHS exposure, compared to the non-smokers with cotinine ≤ 0.05 ng/mL. Active smokers had lower BMI, fasting insulin and albumin compared with non-smokers with cotinine ≤ 0.05 ng/mL. Smokers had higher triglycerides, CRP, IL-6, white blood cell count, fibrin D-dimer, plasma and blood viscosity, fibrinogen, VWF, t-PA, platelet count, homocysteine, CD40 Ligand and MMP-9. The largest percentage differences in CVD risk factor between smokers and non-smokers with cotinine ≤ 0.05 ng/mL were for CRP (62%), IL-6 (41%), MMP-9 (27%) fibrin D-dimer (21%) and white blood cell count (18%). Active smoking was not appreciably associated with blood pressure, waist circumference, total and HDL cholesterol, glucose, mean platelet volume, factor VII, factor VIII, haematocrit, IL-18 or TNF α levels.

Among non-smokers, higher cotinine levels were associated with higher BMI, waist circumference, fasting insulin, CRP, IL-6, blood and plasma viscosity, fibrinogen, factor VIII, VWF, t-PA and with lower albumin (Table 2, Figures 1a-1d). Modest associations with factor VII, triglycerides and platelet count did not reach conventional levels (5%) of statistical significance; there were no associations with BP, fasting lipids and glucose, white cell count, fibrin D-dimer, mean platelet volume, haematocrit, homocysteine, IL-18, CD40L, MMP-9 and

TNF α . Among non-smokers the largest percentage changes in CVD risk factors in participants with cotinine >0.7ng/mL compared to \leq 0.05ng/mL were for CRP (26%), IL-6 (12%) and t-PA (9%).

Table 3 presents associations in non-smokers between log₂[cotinine] and each risk factor in models adjusted first for gender, age and region of residence, then additionally for social and behavioural confounders including smoking history and BMI. The coefficients represent the risk factor difference associated with a doubling in cotinine concentration. Among factors where associations with increasing SHS exposure were in the same direction as associations with active smoking in earlier analyses, on full adjustment, CRP, IL6, fibrinogen, factor VIII, VWF, t-PA and albumin were still associated with cotinine, although adjustments attenuated the strengths of associations by up to half (for CRP, IL-6 and t-PA) and up to a quarter (albumin and factor VIII). Adjusted associations between cotinine level and blood and plasma viscosity were attenuated to the null. Cotinine levels were not consistently associated with BP, lipids, insulin or glucose, white cell count, fibrin D-dimer, mean platelet volume, platelet count, haematocrit, homocysteine, IL-18, CD40 ligand, MMP-9 or TNF α , after adjustment for health behaviours, social class and BMI. Additional adjustments for lipids and BP did not materially affect results except for IL-6, which was attenuated to the null. Treating cotinine as a dichotomous variable, (>0.7 ng/ml compared to \leq 0.05) yielded similar results.

Restricting analyses in Table 3 to never-smokers (n=2496) reduced precision; associations of cotinine with BMI, CRP, IL-6 and albumin were of similar magnitude to those reported above for all current non-smokers but confidence intervals were wide and included the null value.

Associations with waist circumference and fibrinogen were not found in never-smokers. Other significant associations between cotinine and risk markers were not materially changed by

restriction to never-smokers, or to non-smokers with cotinine ≤ 9.5 ng/mL. Variables which were robustly associated with cotinine (Table 3), showed similar associations in both sexes and did not vary by age in regression models with cotinine as a continuous or a dichotomous variable (all LR tests of interactions, $P > 0.05$). Sensitivity analyses using 0.025 and 0.075 rather than 0.05ng/ml for undetectable cotinine gave a similar pattern of results.

DISCUSSION

Several cardiovascular risk factors showed independent associations with SHS exposure in the same direction as active smoking, adjusted for age, gender, social and behavioural factors. These included CRP, fibrinogen, factor VIII, VWF and t-PA (all of which had higher circulating levels both with active smoking and SHS exposure). Although active smokers had higher triglyceride and insulin levels and lower mean BMI and waist circumference than non-smokers with low SHS exposure, these factors did not show similar patterns of associations with SHS exposure in adjusted analyses.

Strengths and weaknesses

This is the first study to examine associations between serum cotinine levels in non-smokers and an exceptionally wide range of established and novel CVD risk factors and benefits from a large population including both sexes sets results in context using active smokers. Serum cotinine is an objective marker that is well validated to measure overall SHS exposure, which has advantages over self-reported exposure (18). This study is unique in investigating a far wider range of risk factors than the two earlier studies with cotinine data (5;19). A limitation is the inability to completely exclude residual confounding of associations between cotinine and CVD markers, particularly by socio-economic factors and adiposity.

Other studies

Our data comparing risk factor levels between active smokers and non-smokers with undetectable cotinine are potentially less biased than those of other studies, which generally included participants exposed to SHS in the non-smoker reference group. However, overall the results are similar in showing that active smokers had lower BMI and waist circumference and albumin compared with non-smokers, but higher triglycerides(13-16), inflammatory, hemostatic and endothelial markers (13;27;28). Our results for SHS exposure, finding associations with biomarkers of inflammation, hemostasis and endothelial dysfunction rather than with established CHD risk factors are consistent with an earlier report relating self-reported SHS exposure of >1 hour/week to higher CRP and white blood cell count, but not to BP or lipids (29), although other studies do report that SHS exposure is associated with lower HDLc(3). Our findings indicating a generalised inflammatory response to SHS exposure fit with prior evidence that CRP is raised (with self-report or cotinine) (17;30), although we did not observe elevated white blood cell counts reported elsewhere (17;19;29). The observed associations between hemostatic factors (several of which are also acute phase reactants) and SHS exposure are consistent with some earlier data. Positive associations between SHS exposure (self-reported or cotinine levels) and fibrinogen levels have been reported previously (19;22). Although associations between active smoking and higher VWF levels have been reported previously (14;15), our study appears to be the first reporting associations with SHS exposure. The association with VWF fits with reports of impaired flow-mediated dilatation, a more direct measure of endothelial dysfunction, in smokers than non-smokers and an inverse dose-response association of poorer dilatation with greater SHS exposure (12). Active smoking is associated with elevated oxidative stress which may in turn alter endothelial function, for instance through reduced nitric oxide (NO) bioavailability, although data were not available here to investigate these pathways for SHS exposure. We did not replicate positive associations between SHS exposure (self-reported or cotinine) and homocysteine (10;17;19), possibly because we had fewer participants with homocysteine data.

Interpretation of results

SHS exposure exhibited graded trends with several, although not all inflammatory, hemostatic and endothelial markers which are also related to active smoking. Selection bias is unlikely given our reasonably high response rates, as is misclassification of active smokers wrongly placed into high SHS group; participants with elevated cotinine (>15 ng/ml) were excluded from SHS analyses. Results are not affected by using a more conservative cotinine threshold for active smoking (9.5ng/ml) (31). Restricting analyses to self-reported never-smokers reduced power and adjusted associations with BMI, CRP, IL-6, albumin and fibrinogen were no-longer significant, bringing into question their causative roles. Residual confounding, particularly by social factors is possible despite statistically controlling for social class. We specifically examined the effect of adjustment for social class after adjusting for behavioural and demographic factors and social class little affected the cotinine coefficient. Observed associations between SHS and endothelial, inflammatory and hemostatic markers were somewhat attenuated by adjustments, although CRP, VWF, tPA, fibrinogen and albumin remained statistically significant after adjustment for social, behavioural and established risk factors plus BMI. It is biologically plausible that associations between SHS exposure and CVD risk markers are causal. The cardiovascular system is very sensitive to SHS and inflammation and platelet activation are prominent in the response to SHS; fibrinogen, VWF and t-PA are markers of both processes (3;19). As in previous studies(19), the influence of SHS on inflammatory and hemostatic markers starts at very low levels of exposure (being apparent well below 0.7 ng/ml, the cotinine level associated with increased CHD risk in our earlier study(5)) and are disproportionate to exposure levels. The magnitude of differences in CRP, fibrinogen and albumin between high and low SHS exposure are around one-third to one half of the magnitude of differences between active smokers and low SHS exposure, although the differences are of similar magnitude for VWF and t-PA. Given current evidence about the strengths of association of CRP, VWF and t-PA to CHD risk (28;32;33), the potential impact of SHS exposure (cotinine ≤ 0.05 vs >0.7) acting through each pathway is approximately 5-10% and

could therefore be expected to affect population levels of CHD. Several of the inflammatory and hemostatic factors have been implicated in causation of type 2 diabetes (34) which would be consistent with an earlier report implicating SHS exposure in type 2 diabetes (35).

Conclusions

This study suggests that SHS exposure, even at very low levels has important influences on endothelial, inflammatory and hemostatic factors. The findings emphasize the continued importance of efforts to reduce exposure to SHS, even at very low levels.

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Table 1. Association between serum cotinine in non and active smokers and demographic or behavioural factors [mean or %]*

| | N | Non-smokers (serum cotinine ng/mL) | | | | Cigarette smokers (1-9 /day) (n=272) | <i>P</i> (trend) [†] in non-smokers | <i>P</i> (no difference) [†] ≤0.05ng/mL vs smokers |
|--|------|------------------------------------|--------------------|--------------------|-----------------|--------------------------------------|--|---|
| | | ≤0.05 (n=1994) | 0.06-0.19 (n=1130) | 0.20-0.70 (n=1034) | 0.71-15 (n=599) | | | |
| Age (years) | 5029 | 69.3 | 68.4 | 68.0 | 67.9 | 69.1 | <0.001 | 0.470 |
| Women (%) | 5029 | 62.5 | 55.0 | 48.7 | 44.4 | 59.9 | <0.001 | 0.310 |
| Northern region of residence (%) | 5029 | 33.2 | 39.5 | 40.8 | 46.1 | 41.9 | <0.001 | <0.001 |
| Manual occupational class (%) | 4735 | 41.2 | 44.9 | 56.3 | 65.7 | 56.3 | <0.001 | <0.001 |
| Light alcohol consumption (1-15 units/week) | 4499 | 39.5 | 43.8 | 45.2 | 39.4 | 39.8 | 0.777 | 0.868 |
| Quit smoking >20 years ago (%) | 4757 | 27.2 | 26.1 | 28.6 | 29.6 | - | 0.770 | - |
| Never smokers (%) | 4757 | 57.2 | 55.2 | 47.7 | 39.7 | - | <0.001 | - |
| Low physical activity (<3 hours moderate/vigorous /week) | 4848 | 62.1 | 59.6 | 59.8 | 60.9 | 73.4 | 0.008 | <0.001 |

* sample excludes participants (i)with prevalent diabetes, stroke or MI, (ii) taking warfarin and (iii) ex-smokers who quit <5 years ago

[†] *P* value from regression model of each factor with log₂[cotinine], adjusted for age, gender and region.

Table 2. Association between serum cotinine in non-smokers and active smokers with cardiovascular risk factors *.

| | | Non-smokers (serum cotinine ng/mL) | | | | Cigarette smokers | <i>P</i> (trend) | <i>P</i> (no difference) [#] |
|--------------------------------------|------|------------------------------------|-----------|-----------|---------|-------------------|--------------------------------|---------------------------------------|
| | N | ≤0.05 | 0.06-0.19 | 0.20-0.70 | 0.71-15 | (1-9/day) | In non-smokers | ≤0.05ng/mL vs smokers |
| | | (n=1994) | (n=1130) | (n=1034) | (n=599) | (n=272) | | |
| Blood pressure | | | | | | | | |
| Systolic (mm/Hg) ^{†‡} | 5000 | 148.82 | 147.74 | 147.31 | 150.08 | 146.23 | 0.774 | 0.070 |
| Diastolic (mm/Hg) ^{†‡} | 5000 | 82.92 | 82.40 | 82.73 | 83.90 | 81.60 | 0.305 | 0.059 |
| Lipids | | | | | | | | |
| Total cholesterol (mMol/L) | 5001 | 6.39 | 6.37 | 6.35 | 6.37 | 6.37 | 0.976 | 0.743 |
| HDL cholesterol (mMol/L) | 4989 | 1.53 | 1.51 | 1.52 | 1.53 | 1.49 | 0.193 | 0.112 |
| Triglyceride (mMol/L) ^{‡§} | 4868 | 1.56 | 1.57 | 1.55 | 1.60 | 1.68 | 0.089 | 0.008 |
| Metabolic Markers | | | | | | | | |
| Body Mass Index (Kg/m ²) | 4488 | 26.70 | 27.11 | 27.34 | 27.37 | 25.62 | <0.001 | <0.001 |
| Waist circumference (cm) | 4394 | 90.22 | 91.35 | 91.58 | 92.06 | 89.10 | <0.001 | 0.160 |
| Insulin (μu/L) ^{‡§} | 4891 | 6.91 | 7.04 | 6.94 | 7.35 | 6.29 | 0.028 | 0.009 |

| | | | | | | | | |
|---|------|--------|--------|--------|--------|--------|--------|--------|
| Glucose (mMol/L) ^{‡§} | 4850 | 5.66 | 5.63 | 5.64 | 5.63 | 5.59 | 0.833 | 0.095 |
| Inflammatory /hemostatic markers | | | | | | | | |
| C-reactive protein (mg/L) [§] | 4961 | 1.41 | 1.49 | 1.60 | 1.78 | 2.29 | <0.001 | <0.001 |
| IL-6 (pg/mL) [§] | 4994 | 2.16 | 2.19 | 2.28 | 2.41 | 3.05 | <0.001 | <0.001 |
| White cell count ([^] 10 ⁹ /L) ^{‡§} | 4793 | 6.60 | 6.61 | 6.60 | 6.66 | 7.76 | 0.175 | <0.001 |
| Albumin (g/L) [‡] | 4868 | 44.33 | 44.20 | 44.08 | 43.87 | 43.59 | <0.001 | <0.001 |
| Fibrin D-dimer (ng/mL) [§] | 5012 | 83.18 | 83.73 | 84.96 | 80.60 | 100.30 | 0.846 | 0.001 |
| Plasma viscosity (mPa.s) [‡] | 4720 | 1.28 | 1.29 | 1.28 | 1.29 | 1.30 | 0.009 | 0.006 |
| Blood viscosity (mPa.s) ^{‡§} | 4624 | 1.55 | 1.56 | 1.56 | 1.57 | 1.58 | 0.007 | 0.006 |
| Fibrinogen (g/L) [‡] | 4875 | 3.19 | 3.18 | 3.22 | 3.28 | 3.49 | 0.026 | <0.001 |
| Factor VII (IU/dL) [‡] | 4868 | 133.51 | 132.89 | 133.07 | 135.82 | 136.00 | 0.052 | 0.172 |
| Factor VIII (IU/dL) | 5011 | 141.72 | 144.08 | 145.09 | 148.83 | 144.63 | <0.001 | 0.173 |
| Von Willebrand Factor (IU/dL) | 5014 | 137.20 | 140.39 | 142.85 | 144.05 | 146.86 | <0.001 | <0.001 |
| t-PA (ng/mL) [‡] | 4880 | 9.26 | 9.43 | 9.70 | 10.04 | 10.05 | <0.001 | 0.001 |
| Mean platelet volume (fL) | 4852 | 8.74 | 8.72 | 8.73 | 8.76 | 8.61 | 0.497 | 0.269 |
| Hematocrit (%) | 4921 | 45.25 | 45.19 | 45.12 | 44.90 | 45.43 | 0.193 | 0.332 |
| Platelet count ([^] 10 ⁹ /L) | 4921 | 246.67 | 245.94 | 248.39 | 251.35 | 262.83 | 0.065 | <0.001 |

| | | | | | | | | |
|------------------------------------|------|--------|--------|--------|--------|--------|-------|--------|
| Homocysteine ($\mu\text{mol/L}$) | 2224 | 12.87 | 13.00 | 12.72 | 13.49 | 14.43 | 0.100 | 0.013 |
| IL-18 (pg/mL) [§] | 1607 | 369.13 | 365.01 | 385.78 | 380.73 | 389.93 | 0.196 | 0.287 |
| CD40 Ligand (pg/mL) [§] | 1614 | 5.42 | 5.23 | 5.32 | 5.61 | 6.09 | 0.318 | 0.029 |
| MMP-9 (ng/mL) [§] | 1614 | 448.93 | 483.14 | 452.90 | 474.47 | 571.03 | 0.219 | <0.001 |
| TNF α (pg/mL) [§] | 1595 | 1.73 | 1.84 | 1.77 | 1.82 | 1.91 | 0.426 | 0.116 |

*sample excludes participants (i)with pre-existing diabetes, stroke or MI, (ii) taking warfarin and (iii) ex-smokers who quit <5 years ago. Means are adjusted for age, gender and region of residence

[†] adjusted for intra-observer variation

[‡] adjusted for time of day

[§] geometric mean

^{||} *P* value for trend from linear regression models in non-smokers of each marker with log₂[cotinine] adjusted for age, gender and region.

[#] *P* value for difference between cotinine ≤ 0.05 ng/mL and smokers (≥ 1 cigarette/day), from logistic regression models of each marker adjusted for age, gender and region.

Table 3. Association between cardiovascular risk factors and serum cotinine in non-smokers. The figures represent the risk factor difference associated with a doubling in cotinine concentration.

| | N | Model 1= age + region + gender | Model 2= Model 1+ health behaviours +SEP +BMI [†] |
|---|------|--------------------------------------|---|
| <i>Blood pressure</i> | | | |
| Systolic (mm/Hg) ^{‡§} | 4273 | -0.04 (-0.46, 0.36) | -0.27 (-0.69, 0.15) |
| Diastolic (mm/Hg) ^{‡§} | 4273 | 0.06 (-0.14, 0.26) | -0.02 (-0.23, 0.18) |
| <i>Lipids</i> | | | |
| Total cholesterol (mMol/L) | 4277 | -0.00 (-0.02, 0.02) | -0.00 (-0.02, 0.02) |
| HDL cholesterol (mMol/L) | 4266 | -0.00 (-0.01, 0.00) | -0.00 (-0.01, 0.01) |
| Triglyceride (mMol/L) [§] | 4176 | 0.00 (-0.00, 0.01) | -0.00 (-0.01, 0.01) |
| <i>Metabolic Markers</i> | | | |
| Body Mass Index (Kg/m²) | 4295 | 0.16 (0.09, 0.23) | 0.11 (0.04, 0.19) |
| Waist circumference (cm) | 4203 | 0.44 (0.25, 0.63) | 0.27 (0.08, 0.46) |
| Insulin (µu/L) [§] | 4192 | 0.01 (-0.00, 0.02) | 0.00 (-0.01, 0.01) |
| Glucose (mMol/L) [§] | 4162 | 0.002 (-0.004, 0.000) | -0.002 (-0.005, 0.000) |
| <i>Inflammatory /hemostatic markers</i> | | | |
| C-reactive protein (mg/L) | 4241 | 0.05 (0.03, 0.07) | 0.03 (0.01, 0.05) |
| IL-6 (pg/mL) | 4267 | 0.03 (0.02, 0.04) | 0.01 (0.00, 0.02) |
| White cell count (^10 ⁹ /L) [§] | 4116 | 0.00 (-0.00, 0.01) | 0.00 (-0.00, 0.01) |
| Albumin (g/L)[§] | 4176 | -0.09 (-0.14,-0.05) | -0.07 (-0.12, -0.03) |
| Fibrin D-dimer (ng/mL) | 4282 | 0.00 (-0.01, 0.01) | -0.01 (-0.02, 0.01) |
| Plasma viscosity (mPa.s) [§] | 4053 | 0.002 (0.001, 0.004) | 0.001 (-0.000, 0.003) |
| Blood viscosity (mPa.s) [§] | 3978 | 0.003 (0.001, 0.004) | 0.002 (-0.000, 0.003) |
| Fibrinogen g/L[§] | 4176 | 0.02 (0.00, 0.03) | 0.02 (0.00, 0.03) |
| Factor VII (IU/dL) [§] | 4170 | 0.44 (-0.08, 0.96) | 0.06 (-0.47, 0.59) |

| | | | |
|---|------|--------------------------|--------------------------|
| Factor VIII (IU/dL) | 4279 | 1.41 (0.81, 2.00) | 1.09 (0.48, 1.69) |
| Von Willebrand Factor (IU/dL) | 4283 | 1.98 (1.20, 2.76) | 1.75 (0.95, 2.54) |
| t-PA (ng/mL)[§] | 4182 | 0.17 (0.11, 0.24) | 0.08 (0.02, 0.15) |
| Mean platelet volume (fL) | 4147 | 0.01(-0.02, 0.04) | 0.00 (-0.03, 0.03) |
| Hematocrit (%) | 4212 | -0.05 (-0.11, 0.01) | -0.06 (-0.12, -0.00) |
| Platelet count ($\times 10^9/L$) | 4212 | 1.10 (-0.02, 2.24) | 0.93 (-0.20, 2.10) |
| Homocysteine($\mu\text{mol/L}$)(men only) | 2024 | 0.11 (-0.03, 0.24) | 0.06 (-0.08, 0.20) |
| IL-18 (pg/mL) | 1349 | 0.01 (-0.01, 0.02) | 0.01 (-0.01, 0.02) |
| CD40 Ligand (pg/mL) | 1355 | 0.01 (-0.01, 0.03) | 0.01 (-0.01, 0.03) |
| MMP-9 (ng/mL) | 1355 | 0.01 (-0.01, 0.03) | 0.01 (-0.01, 0.03) |
| TNF α (pg/mL) | 1340 | 0.01 (-0.01, 0.03) | 0.01 (-0.01, 0.03) |

* Results in bold reach conventional statistical significance levels (5%).

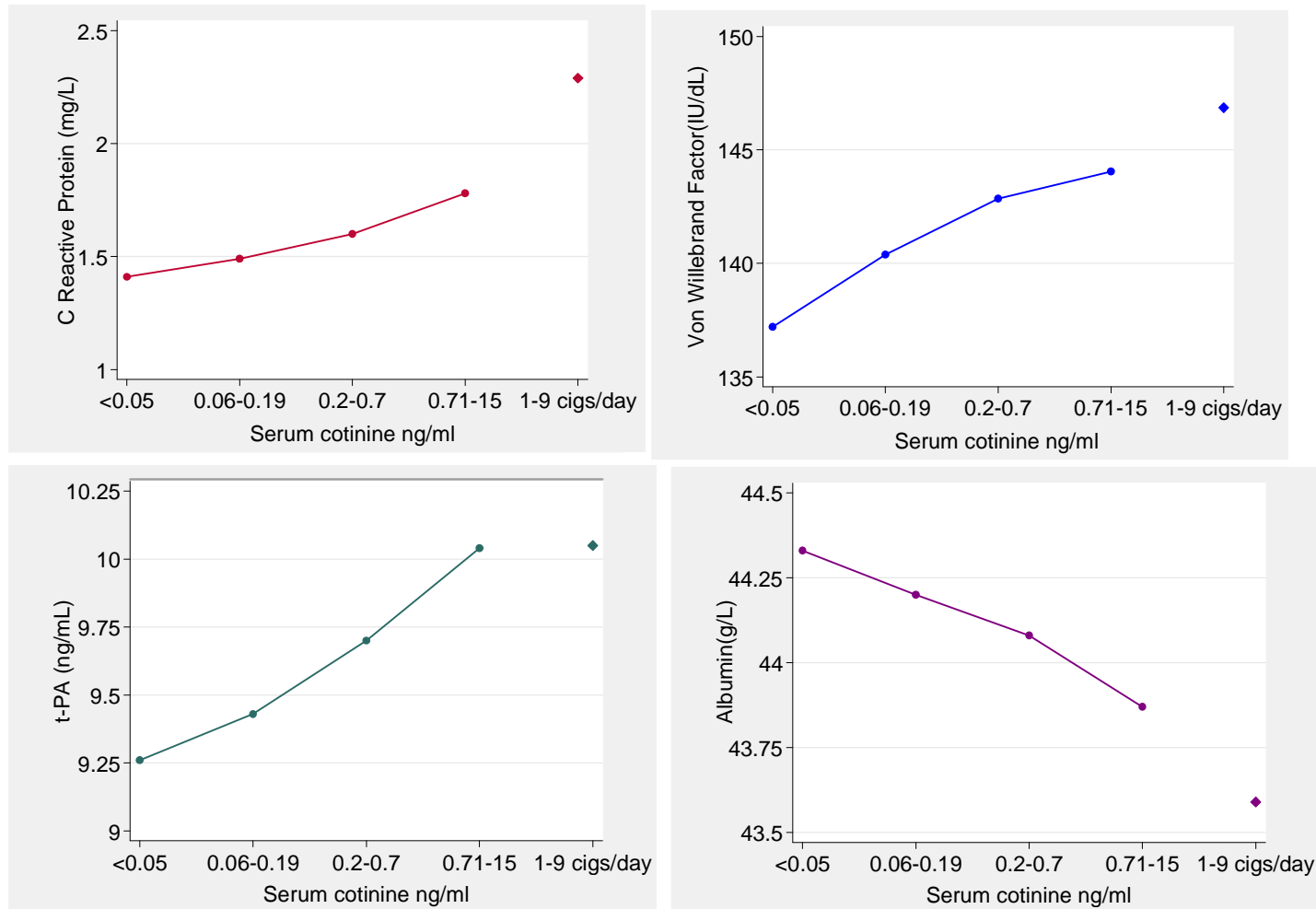
[†]adjusted for alcohol intake (6 categories), smoking history (5 categories), physical activity (inactive/active), SEP(manual, non-manual, armed forces), BMI (and BMI*gender where appropriate). Waist circumference not adjusted for BMI.

[‡]adjusted for intra-observer variation

[§]adjusted for time of day

^{||}natural log transformed: coefficient reported for log variable represents a proportional change

Figure 1 Associations between serum cotinine level in non-smokers, or active smokers of 1-9 cigarettes/day and (a) CRP (b) VWF (c) t-PA (d) Albumin



Supplementary data

METHODS

Study design:

In 1998-2000, 4252 men from a single General Practice in each of 24 British towns who were already participating in a prospective study of cardiovascular disease attended for follow-up measurements at the age of 60-79 years; response rate 77% (1). In 1999-2001, a parallel study of 4286 women of the same age and in the same practices was established, with the addition one study town (Bristol) and the omission of two others (Dewsbury and Maidstone), response rate 60% (2). Near identical protocols for data collection were used. Participants completed questionnaires including detailed questions about current and previous smoking history (cigarettes, pipes and cigars), alcohol use, physical activity, occupation, and medication use. Social class was based on longest held occupation of each man in 1978-80, classified using Registrar General scale; I, II and III non-manual compared to III manual, IV and V. Men in the Armed Forces were classified separately. Class was based on own occupation for single women and on husband's longest held occupation for married, widowed or divorced women. Region of residence at baseline was defined as Scotland, North, Midlands and South as previously reported (2;3). Nurses measured height, weight and BP, reported elsewhere(4), recorded an ECG and collected fasting venous blood samples, from which plasma serum was stored at $\leq -70^{\circ}\text{C}$.

Laboratory assays

Serum samples were assayed for cotinine in 2007-2008 at ABS Laboratories Ltd, London UK. For non-smokers a liquid chromatography tandem mass spectrometry (LC MS/MS) assay with a lower limit of detection of 0.02 ng/mL with a limit of quantification of 0.1 ng/mL was used which is a modification of the published GC-NPD method (5) Cotinine values at the limit of quantification were assigned a value of 0.05ng/ml, the mid point between 0.1 and zero, as in other

studies (6) and sensitivity analyses (using values of 0.25 and 0.75ng/ml), assessed the validity of this assumption. Assays for blood lipids and metabolic markers (HDLc, total cholesterol, triglycerides, insulin and glucose) a range of inflammatory (CRP, IL-6, white blood cell count, albumin, plasma and blood viscosity, Interleukin-18, Matrix Metalloproteinase-9 and Tumor Necrosis Factor α) and hemostatic markers (fibrinogen, D-dimer, von Willebrand Factor, tissue plasminogen activator, Factor VII, Factor VIII, platelet size, platelet count and homocysteine) are described elsewhere(4;7-10). Soluble CD40-Ligand was measured with a commercially available ELISA kit (R&D systems, Oxon, UK). Intra-assay variation was <5% and inter-assay variation was <10%.

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